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Award Number:
Contract W81XWH-09-1-0715

TITLE:
Applying Genomic and Genetic Tools to Understand and Mitigate Damage from Exposure to Toxins

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REPORT DATE:
October 2013

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Final	21 September 2009 - 20 September 2013

4. TITLE AND SUBTITLE
Applying genomic and genetic tools to understand and mitigate damage from exposure to toxins

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0715

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The use of pyridostigmine bromide (PB) in the Gulf War of 1991 has been associated with increased incidence and symptom severity of Gulf War Illness. The goal of our research program was to use zebrafish as a model organism to characterize the effects of PB using locomotive/behavioral phenotyping and unbiased, high-throughput techniques, specifically mRNA-seq. Fish treated with PB exhibited perturbed behavioral patterns as indicated by changes in place preference. Exposure to PB altered expression of genes related to the synapse, calcium binding and signaling, cytoskeleton, cell junctions, oxidation-reduction reactions, and regulation of transcription in both larval and adult zebrafish. We studied the impact of genetic background using three strains of zebrafish. We also investigated the effect of stress on the PB response in both larval and adult zebrafish. The study of adult zebrafish allowed for characterization of tissue-specific gene expression in the brain and skeletal muscle, both immediately following PB exposure and at 8 weeks thereafter. These findings provide new insight into the effects of PB at the molecular level, which may aid in the development of therapies for Gulf War Illness.

15. SUBJECT TERMS
pyridostigmine bromide, Gulf War Illness, zebrafish

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	35	19b. TELEPHONE NUMBER *(include area code)*

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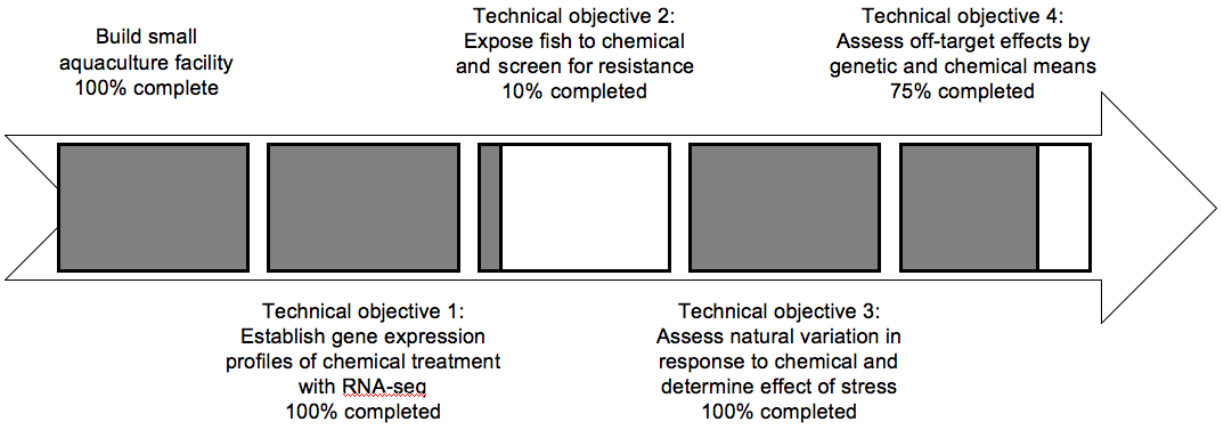
EXECUTIVE SUMMARY

Soldiers of the 1991 Gulf War were given the drug pyridostigmine bromide (PB) for pretreatment against nerve agents. This drug has since been associated with increased incidence and symptom severity of Gulf War Illness. The goal of our research program was to use zebrafish as a model organism for the characterization of the effects of pyridostigmine bromide on gene expression using unbiased, high-throughput techniques, specifically mRNA-seq. Additionally, we intended to study the PB response in the presence of factors that may modulate the effects of PB as indicated by epidemiological and experimental evidence, such as genetic variation or stress. Over the duration of the award, we have successfully characterized phenotypes associated with PB exposure and measured the molecular response using mRNA-seq. We further studied the effects of PB in the context of three different genetic backgrounds and under stressful conditions. Larval zebrafish were utilized to study the systemic response, whereas results from experiments with adult fish provided valuable knowledge of effects that were tissue-specific and those that were observed 8 weeks after a short-term exposure. Two strains of acetylcholinesterase (AChE) mutant zebrafish were used as a genetic model of lowered levels of AChE activity and two other AChE inhibitors were studied for comparison to the effects to AChE inhibition by PB. These experiments laid the foundation for studying the off-target effects of PB and for consideration of therapeutic alternatives to PB, respectively. With the completion of this research, we have provided insight into the predominant changes in gene expression in response to PB and related these changes to the phenotypes of PB-exposed zebrafish. We anticipate that a greater understanding of the effects of PB at the molecular level will inform decisions regarding the safety and use of PB and aid in the development of therapies for veterans afflicted by Gulf War Illness.

Contract No. W81XWH-09-1-0715 / Proposal #08273008
HudsonAlpha Institute for Biotechnology
Richard M. Myers, PI
Final report: 21 Sept 2009-20 Sep 2013

Progress and expected milestones:



INTRODUCTION

Pyridostigmine bromide (PB) was given to approximately 250,000 soldiers of the 1991 Gulf War as a prophylactic against potential nerve agent attack (*1*). Pyridostigmine is a peripheral acetylcholinesterase (AChE) inhibitor that reversibly binds AChE, thereby protecting the enzyme from being bound by nerve agents, which are irreversible AChE inhibitors. Use of PB has since been positively associated with an increased incidence of Gulf War Illness and increased severity of the symptoms of Gulf War Illness, such as fatigue, musculoskeletal pain, mood and cognitive disorders, dermatological irritation, and gastrointestinal effects (*2*). Recent epidemiological evidence suggests that for soldiers in the most forward deployments, PB exposure was the greatest factor associated with Gulf War Illness (*3*). Research also suggests that there are potential interactions between the effects of PB and additional factors, specifically stress and genetics. Although the precise mechanism is unclear, treatment with PB concurrent with stressful conditions has been shown to result in aberrant behavioral patterns and altered gene expression, enzyme activities, and/or reactive oxygen species levels in the brain and muscle (*4-6*). Genetic variation has also been associated with poor health in Gulf War veterans. For example, variants of paraoxonase 1 and butyrylcholinesterase that result in slower detoxification of organophosphates and PB have been found to be associated with an increased likelihood of Gulf War Illness symptoms in veterans (*7, 8*). Therefore, it was our objective to study the effects of PB alone and in the context of stress and varied genetic backgrounds by using zebrafish as a model organism and utilizing the high-throughput technology of mRNA-seq.

BODY

The goal of our research program (W81XWH-09-1-0715) was to utilize genetic and genomic tools to determine the effects of PB in order to further understand the pathogenesis of Gulf War Illness. Over the duration of the award we completed construction of an aquaculture facility and advanced knowledge related to the following objectives: i) establishment of gene expression profiles of chemical treatment with RNA-seq, ii) determination of the influence of natural variation and stress on the response to chemical, iii) assessment of off-target effects by genetic and chemical means, and iv) exposure of fish to chemical with screening for resistance.

Construction of an aquaculture facility

Construction of the aquaculture facility at HudsonAlpha necessitated remodeling of a room including refinishing of the floor and reconfiguration of drains and electrical work. Supplies were ordered and the aquaculture facility was completed with the installation of the fish racks and water system taking place June 28-29, 2010. The final inspection by the HudsonAlpha IACUC took place on July 1, 2010. All personnel that have had direct contact with the animals completed the Working with Laboratory Zebrafish and Working with the IACUC training modules provided by the American Association for Laboratory Animal Science. Following inspection and training, final approval for animal protocols was received from the HudsonAlpha IACUC and ACURO on July 9 and July 26, 2010 respectively. The first fish were received from the Zebrafish International Resource Center on August 12, 2010. The facility houses 5 strains of zebrafish including the AB and TU strains, which are robust and commonly-used wild type fish, and the SJD strain, which is not fully inbred, but tends to have less genetic variability and may be more amenable for genetic and genomic experimentation. We also acquired the *ache*^{tf222a}

and *ache*^{tm205} mutant strains, which were utilized as a reference for the phenotype associated with lowered levels of AChE activity.

Technical objective 1: establishment of gene expression profiles of chemical treatment with RNA-seq

Dose-response and time-course experiments in larval and adult zebrafish were conducted to determine alterations in gene expression profiles in response to PB exposure. In addition to generating preliminary data on the effects of PB, these studies were essential for identifying the optimal dosage and duration of treatment for subsequent experiments.

During the Gulf War, PB was administered in blister packs of 21 pills, with each pill containing a 30 mg dose of PB to be taken every eight hours while the threat of nerve attack persisted (*1*). This dosage is expected to maintain sustained inhibition of AChE activity by approximately 30%. To examine the dose-response to PB in larval zebrafish and identify the proper dose for subsequent experiments, we conducted a study in which pooled TU zebrafish larvae (n = 20) were treated with solutions of 0, 0.01, 0.1, 1, or 10 mmol PB/L (n = 4 per treatment) from 4-7 days post-fertilization (dpf), the period immediately following completion of organogenesis and hatching of the embryo. Following treatment, the larvae were rinsed three times with ice-cold phosphate-buffered saline (PBS) and snap frozen on dry ice. Ten larvae were preserved in Trizol for extraction of nucleic acids for mRNA-seq, while the remaining larvae were used for preparation of homogenates for enzymatically-active protein using the PARIS Kit (Ambion, Inc.). Phenotypes associated with the primary action of PB, altered acetylcholine signaling, were characterized by determination of AChE activity levels and assessment of body posture following movement elicited by the startle response. AChE activity was determined using the AmplexRed Acetylcholinesterase Assay (Invitrogen, Inc.). Individual zebrafish larvae were treated with 0, 0.01, or 10 mmol PB/L (n = 4 per treatment) for 72-h and movement was video recorded during trials in which the startle response was elicited ten times by approaching the larvae with a blunt object. The angle of the tail bend at rest following the startle response was measured as an indicator of the neuromuscular effects of PB.

Inhibition of AChE activity was dose-dependent in larval zebrafish, with doses of 0.1 and 1 mmol PB/L producing levels of AChE inhibition that were within the therapeutically relevant range of ~20-50% (**Figure 1**). We also observed neuromuscular effects of PB in fish treated with 10 mmol PB/L as evidenced by a greater angle in the tail bend at rest following the startle response in zebrafish larvae (**Figure 2,** p = 0.01). The increased bend of the tail suggests increased contractile tension, which may be due to hyperstimulation of acetylcholine receptors in response to inhibition of the AChE activity and the resulting accumulation of acetylcholine at the

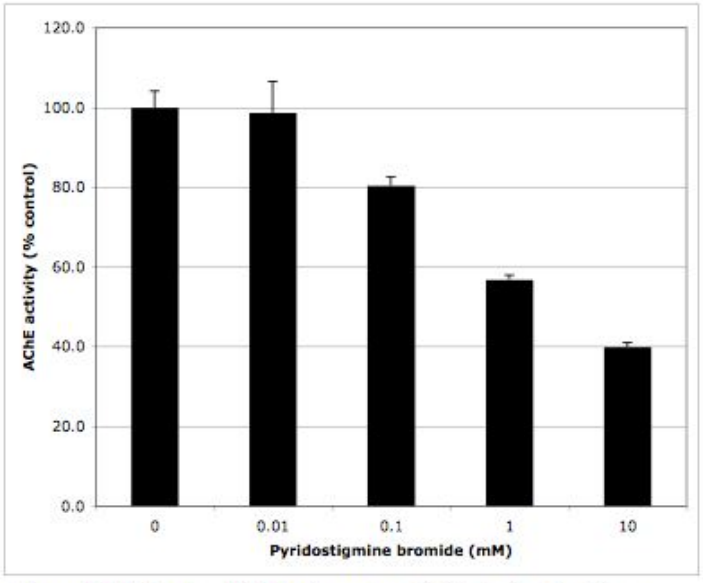


Figure 1. Inhibition of acetylcholinesterase by pyridostigmine bromide is dose-dependent in larval zebrafish. Data are means (n = 4) ± s.e.

neuromuscular synapse. These data indicate that zebrafish larvae are an appropriate model for the study of PB: we were able to achieve levels of inhibition similar to that used therapeutically; and we observed neuromuscular effects at a higher dose, which is in line with the results that have been reported previously in other species (*1*).

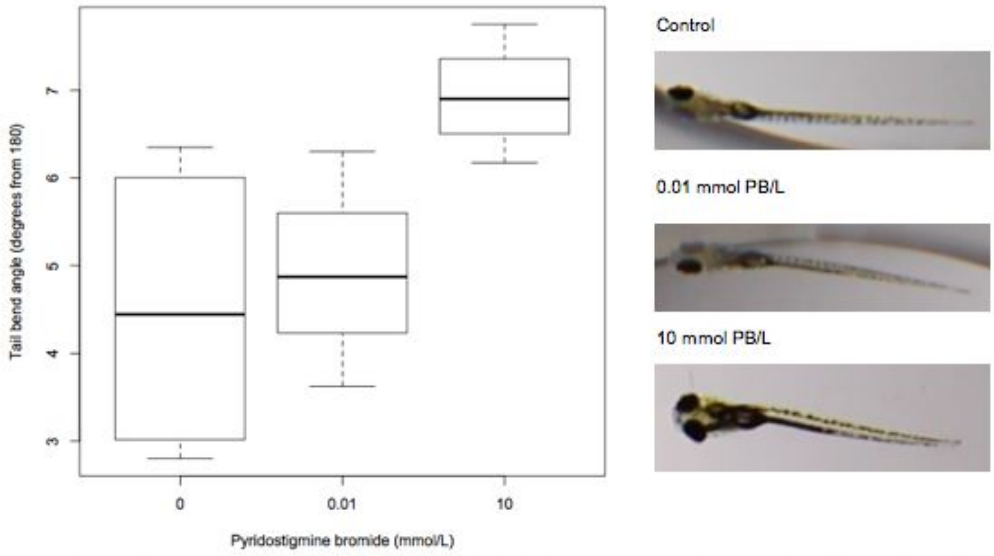


Figure 2. 72-hr treatment of TU larval zebrafish with pyridostigmine bromide produces subtle phenotypic effects after the startle response.

Preliminary mRNA-seq data from two samples per treatment dose was produced to assess the effects of PB at the molecular level. RNA isolated using the Trizol protocol was used as input for the generation of libraries via a protocol developed by our lab that uses Nextera technology (*9*). The libraries were sequenced on an Illumina HiSeq and the resulting sequence was aligned to zebrafish RefSeq exons (zv9) using Bowtie (*10*). Variance stabilization of the data and differential expression for each PB-treated group compared to controls were calculated using statistical methodologies developed in our lab, as described by Reddy *et al* (*11*). This analysis identified 81 transcripts differentially expressed in response to 10 mmol/L PB treatment when compared to control larvae; there were four and three differentially expressed transcripts in response to the 1 and 0.1 mmol/L PB treatments, respectively. The DAVID Functional Annotation Tool (*12, 13*) was used to test for functional annotation enrichments within the differentially expressed transcripts at 10 mmol PB/L, with significant enrichment assigned to clusters with scores greater than or equal to 1.30 or terms meeting an FDR cutoff of 5. The 44 upregulated transcripts were significantly enriched for hemopexin/matrixin repeat, peptidase activity, and endopeptidase inhibitor activity, with data also suggestive of enrichment for heme binding (FDR = 7.15). The 37 downregulated transcripts were significantly enriched for cognition and sensory perception, intermediate filament proteins, EF-HAND and calcium binding, with data suggestive of enrichment for the KEGG pathway related to biosynthesis of unsaturated fatty acids (FDR = 8.98).

The order-restricted inference for ordered gene expression (ORIOGEN) software (*14*) was used to look for dose-responsive genes. Although we were unable to obtain statistical significance at the genome-wide level with this small preliminary data set, enrichment of the genes for which a significant dose-response was found at the gene level ($p < 0.05$) identified terms that appear plausible based on the known molecular action of PB. At the gene-level, 946 genes were found to have an increasing profile and 597 genes had a decreasing profile. Decreasing transcripts were enriched for terms related to transmission of nerve impulses, neuron projection

development, calcium channels and binding proteins, intermediate filament, immunoglobulin, and metabolism. Increased transcripts were enriched for peptidase activity, GTP binding, transmembrane proteins, cell cycle, interferon regulatory factor, protein localization, FAD-dependent pyridine nucleotide-disulphide oxidoreductase, and endoplasmic reticulum. The results of both analyses are indicative of PB having molecular effects related to its primary mechanism of action, as evidenced by enrichment for terms associated with the synapse and regulation of calcium levels. However, there was also enrichment for terms related to structural components of the cell, the immune response, and peptidase activity that may also be informative.

The results of the dose response study provided valuable information regarding the appropriate dosage to mimic therapeutic conditions. There is epidemiological evidence that consumption of an increased number of PB pills was associated with increased risk of Gulf War Illness and severity of symptoms (*2, 15*). We designed a study to examine the effects of PB treatment after exposure times ranging from 4 hours to 7 days, based on a report that of the soldiers that recalled using PB, the majority took PB for less than seven days (*16*). Pooled TU zebrafish larvae (n = 20) were exposed to 0.5 mmol/L PB solution for 4, 24, 72, or 168-h beginning at 4 dpf; system water was used as a control. Sample preparation, AChE activity assessment, and mRNA-seq were performed as described for the dose-response study.

Our analysis of the effects on AChE activity took into account the exposure to PB, the duration of exposure and a possible interaction between PB and exposure time. Pyridostigmine significantly inhibited AChE activity ($p < 0.01$), however there was no effect of time or an interaction between PB and the duration of exposure (**Figure 3**). Three samples per treatment were prepared for mRNA-seq; sequencing generated ~18-28 million reads aligning to exons of the zebrafish transcriptome. Analysis using a linear regression model with terms for PB treatment, time and the treatment x time interaction revealed a significant effect of time, with 7,322 transcripts identified as differentially expressed. These transcripts were enriched for broad categories such as DNA metabolism, homeostatic process and cell cycle. In this model, there was not a significant effect of PB treatment nor a treatment x time interaction for any transcripts. Data for each time point was analyzed separately (control vs. PB-treated), identifying 136, 327, 980, and 460 DE genes at 4, 24, 72, and 168 hours respectively. At four hours, there was significant enrichment for DNA-dependent DNA replication and DNA metabolic processes. At 24 hours, there was significant enrichment for response to metal ion or inorganic substance, basic-leucine zipper transcription factor, and WD40 repeat. At 72 hours, there was significant enrichment for cytoskeleton, proteasome complex, methylation, and endoplasmic reticulum. At 168 hours, there was significant enrichment for establishment of protein localization, cell redox homeostasis, intracellular signaling cascade, and endoplasmic reticulum.

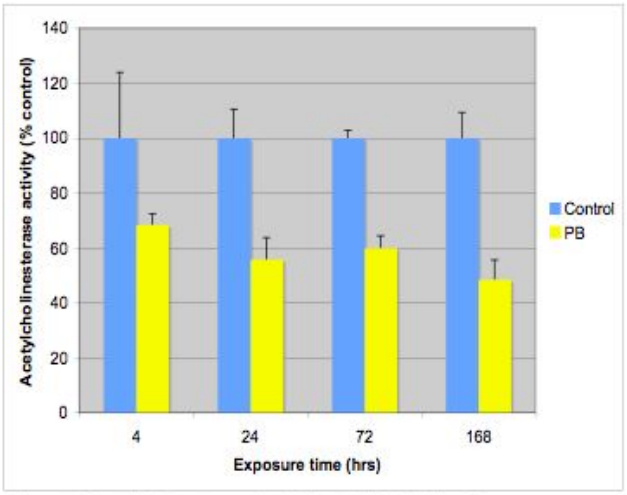


Figure 3. Acetylcholinesterase is significantly inhibited by PB exposure, whereas there is no effect of time. Data are means (n = 3) ± s.e.

The dose response and time course studies provided valuable data regarding the response of larval zebrafish to PB at the phenotypic and molecular levels. Previous studies have suggested that the response to pharmaceuticals or toxins is very similar in larvae and adults when using zebrafish as a model organism (*17*). However, to verify this result for PB, we conducted a preliminary study of the response to PB in adult TU zebrafish. Adult zebrafish (8 months of age) were removed from the communal tank and placed into individual beakers with 300 mL of fish water and allowed to acclimate for 3 days before the initiation of a 24-h treatment with solutions of 0, 0.1, 1, 10, 100, or 1000 μmol PB/L. One male and one female fish were treated at each dose. At the end of the treatment period, the fish were euthanized by submersion in ice water followed by decapitation with a razor blade. The head and the body of each fish were separately prepared for isolation of protein and RNA for the measurement of AChE activity and mRNA-seq by the methods as described for the larval studies. Alignment to the RefSeq exons of the zebrafish genome resulted in 21-48 million reads per sample for downstream analyses.

Consistent with reports that PB is unable to cross the blood-brain barrier (*18*), there were no significant effects of PB treatment on AChE activity in the head. However, AChE activity in the body was significantly inhibited in adult zebrafish treated with either 10 or 1000 μmol PB/L relative to controls (**Figure 4**). Taken together with observations that the dose-response to AChE activity was much stronger in the male fish than the female fish (R^2 = 0.71 vs. 0.14), we chose to do mRNA profiling by mRNA-seq using samples from the heads and bodies of the male fish treated with 0, 10, and 1000 μmol PB/L. Using unsupervised hierarchical clustering based on the 293 most variable genes, samples clustered first by the region of the body (**Figure 5**). Within the tree node representing the body samples, the PB-treated samples clustered away from the control, whereas the differences were much smaller among the samples from the head, with no separation of the PB-treated samples. Our statistical analysis of all expressed genes confirmed that the largest effects were due to the region from with the sample originated, with 2,884 transcripts being differentially expressed between the head and the body (FDR < 0.05). As expected, transcripts that were significantly differentially expressed with at least 50% greater expression in the head were enriched for genes associated with the eyes and brain, whereas the enriched ZFIN anatomy terms associated with greater expression in the body included trunk musculature and the whole organism. Likely due to current lack of replicates, there were no transcripts that met this strict criteria (FDR < 0.05) for the main effect of PB treatment or for the treatment:tissue interaction. However, 407 mRNAs were affected by PB treatment and 1086 mRNAs had a response to PB that also depended on the origin of the sample at the gene-level ($p < 0.05$). Enrichment of terms for the PB response

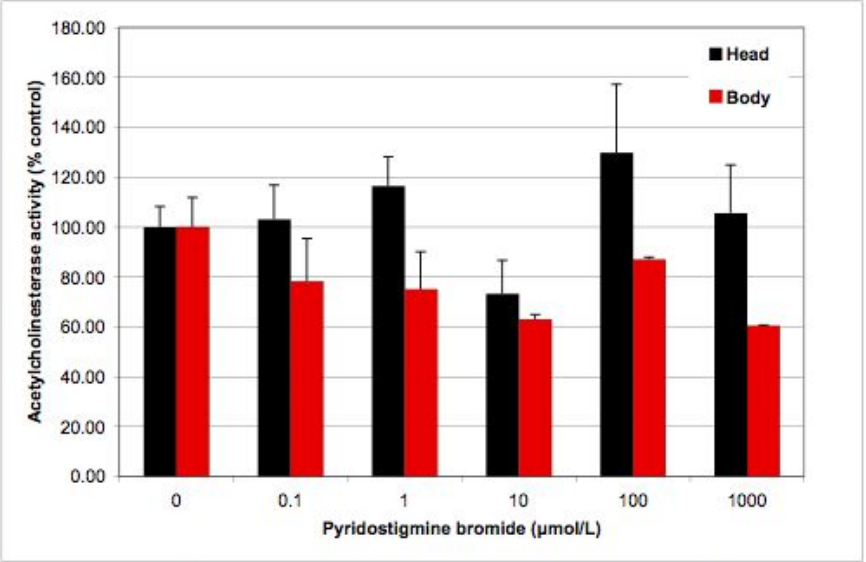


Figure 4. Acetylcholinesterase activity in the head or body of adult zebrafish treated with pyridostigmine bromide for 24-h. Data are means ± s.e.

included calcium ion binding, regulation of peptidase activity, positive regulation of macromolecular biosynthetic process, and sphingolipid metabolism. Enrichment of terms for the PB by tissue interaction included DNA metabolic processes, cell cycle, cytoskeleton, cell projection part, sensory organ development, DNA replication, DNA-dependent ATPase activity, tetrtricopeptide region, DNA binding, and IQ-calmodulin binding region. In an analysis of the samples from the head or the body for the effects of PB separately, there were 893 transcripts differentially regulated by PB in the body and 639 transcripts identified as differentially expressed in the head. Terms enriched in body DE genes included transcription factor activity, microtubule cytoskeleton, sphingolipid metabolism. Terms enriched in the head DE genes included transcription factor complex, ribosome, endosome, intracellular non-membrane-bounded organelle, positive regulation of gene expression (protein heterodimerization activity and nuclear translocation), sensory organ development, ferritin, and chromosome localization.

These studies laid the foundation for the more complex studies of the PB response within the context of other factors in both larval and adult zebrafish. In addition to establishment of the appropriate dose and duration for the PB treatments, these preliminary mRNA-seq data sets were suggestive of PB effects at genes related to the synapse and regulation of calcium levels, as well as additional targets related to cytoskeletal structures, regulation of protein digestion and degradation, and regulation of cellular homeostasis, particularly redox and iron ion homeostasis.

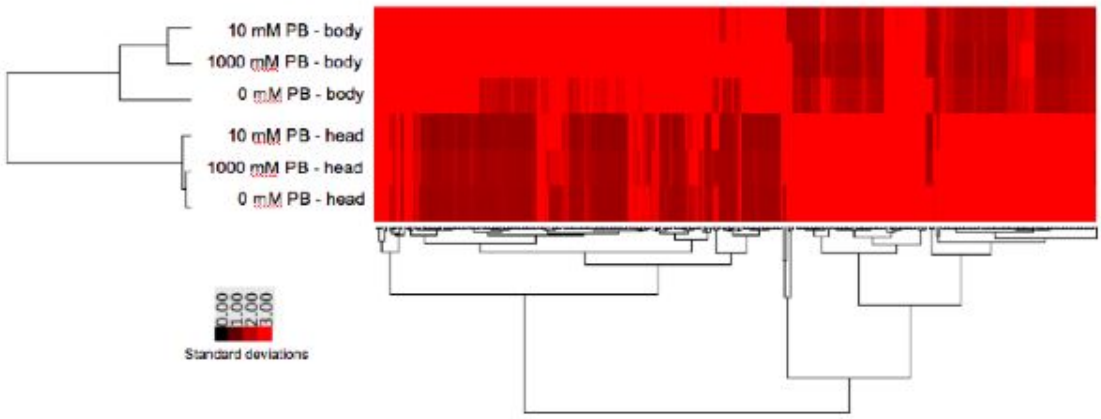


Figure 5. Unsupervised hierarchical cluster of RNA-seq data from the head and bodies of adult zebrafish treated with pyridostigmine bromide.

Technical objective 2: determination of the influence of natural variation and stress on the response to chemical

Effects of genetic variation on the response to pyridostigmine bromide

We conducted an experiment with the goal of further characterizing the dose-dependent effects of PB as well as determining the effect on natural genetic variation on the response to the drug. Pooled groups of 20 larvae from each of three common laboratory strains – AB, TU, and SJD – were treated with solutions of 0, 0.001, 0.01, 0.1, 1, or 10 mmol PB/L for 72-h. The treatment period corresponded to the period of 4-7 days post-fertilization. At 7 dpf, larvae were euthanized and samples were prepared as described for previous larval studies.

Phenotypes associated with PB treatment were assessed by determination of acetylcholinesterase activity and by using motility and neurobehavioral assays. Acetylcholinesterase (AChE) activity was measured by the Amplex Red Acetylcholinesterase Assay (Invitrogen) for determination of the efficacy of treatment and to identify the clinically relevant doses. Pyridostigmine bromide treatment significantly inhibited AChE activity in a dose-dependent manner in all three strains of zebrafish. There was no significant effect of strain nor a treatment by strain interaction. The dose-dependent effect of PB exposure produced inhibition at levels significantly different from the control at all doses greater than or equal to 0.01 M/L (**Figure 6**). For the motility analysis zebrafish larvae were placed in 24-well plates (1 fish per well) and the PB and control solution treatments were randomized across each plate (**Figure 7**). A total of four plates per strain were used for this experimental series, with two plates receiving the stress treatment and two plates serving as controls. Patterns of movement were recorded and quantified using the ZebraLab equipment and VideoTrack software (Noldus Technologies). The protocol tracked movement for five minutes under normal light conditions, an abrupt transition to five minutes of darkness, and an additional five minutes under normal light conditions post-stimulus. Movement patterns in the whole well and the center of the well (50% of total area) were used for determining patterns in total locomotion as well as place preference, both of which can be used as a behavioral indicator of anxiety (*19*). This assay is similar in concept to the open field test used in rodents and is very similar to a protocol that has recently been published by Schnorr *et al.* (*20*), although our protocol also includes characterization of post-stimulus behavior.

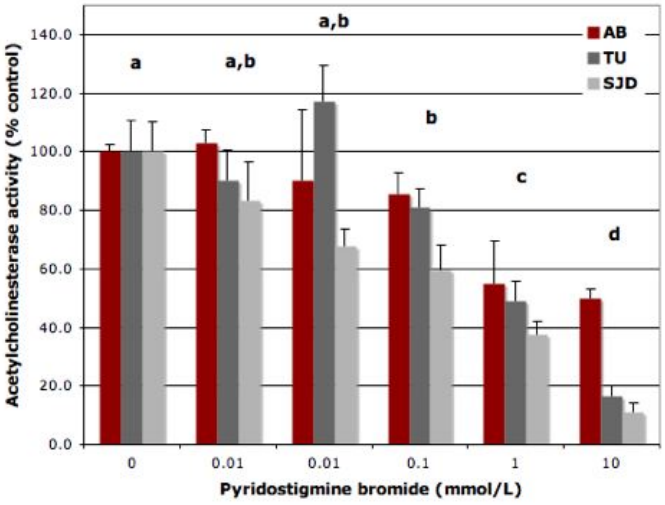


Figure 6. Pyridostigmine bromide inhibits acetylcholinesterase activity in three strains of zebrafish in a dose-dependent manner. Data are means (n = 3) ± s.e.

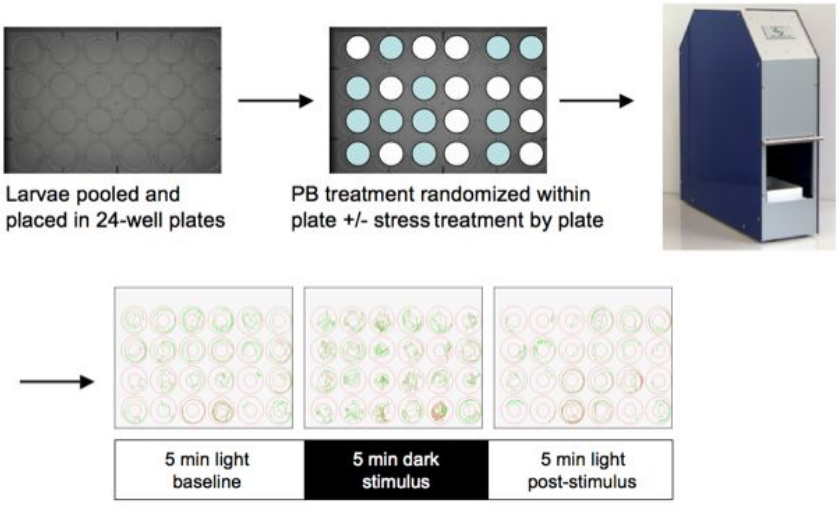


Figure 7. Motility and behavioral analysis of zebrafish larvae under baseline conditions and the response to abruptly changing light conditions.

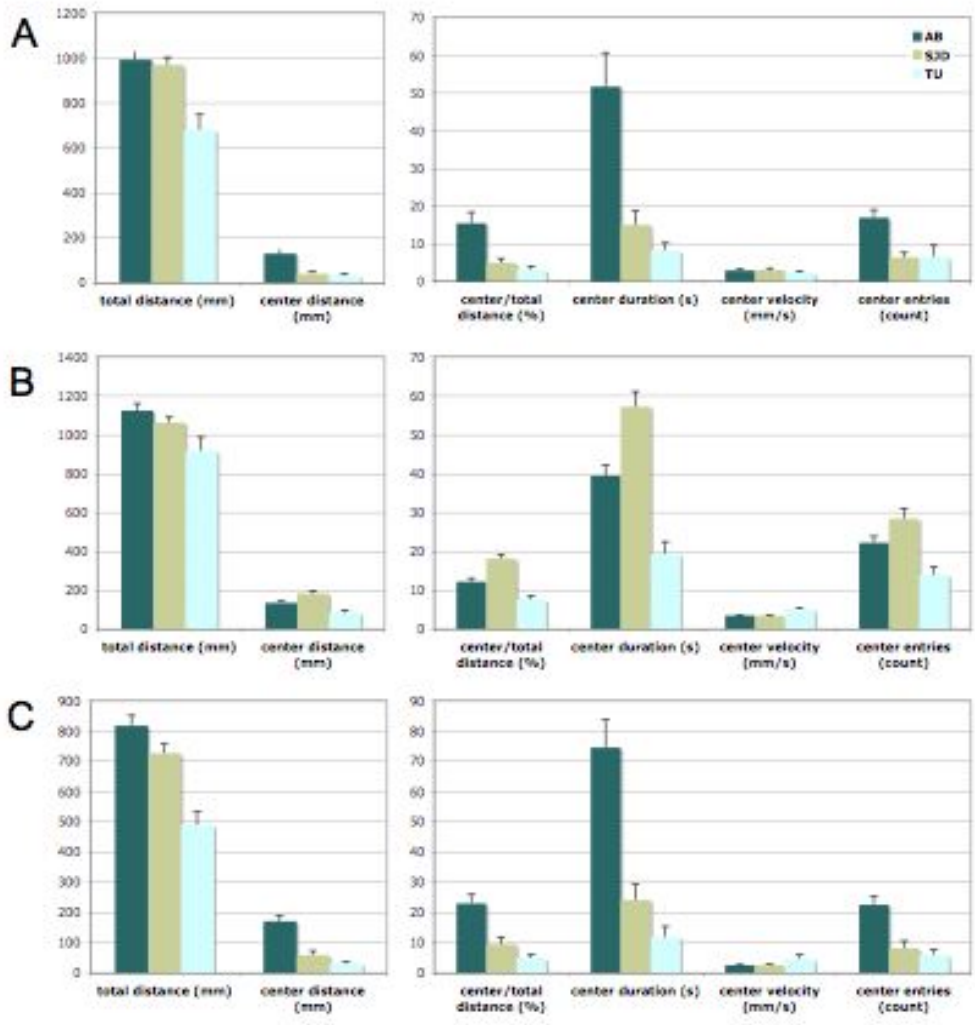


Figure 8. Strain-specific differences in motility and place preference during (A) baseline light phase, (B) dark stimulus, and (C) post-stimulus light phase. Data are means (n = 47-48) ± s.e.

There were a number of differences in locomotion and place preference noted between strains, with differences occurring during all phases of the assay (**Figure 8**). PB treatment resulted in decreased distance traveled in the center at baseline and during the dark phase, decreased total duration in center during dark (trend at baseline), decreased percentage of distance traveled in center during dark (trend at baseline), as well as a trend towards fewer entries into center at baseline (**Figure 9**). A treatment by strain interaction was observed for total distance at baseline, distance and percent total distance in center after stimulus. Analysis of each strain individually for strain-specific effects of PB identified the center distance and duration (alone and as percent of total) significantly decreased in dark for the AB strain, no differences for the SJD strain, and in the TU strain, there were trends for decreased total distance, duration, and average velocity in the center at baseline, as well as a decreased number of center entries in dark. In concordance with data from another study utilizing the light:dark test in assessment of chemical response (ethanol) in different strains of zebrafish (*21*), we also found that there were significant differences in the movement patterns of the three strains of zebrafish, but despite these differences, we were able to detect a response to PB treatment. The distance and

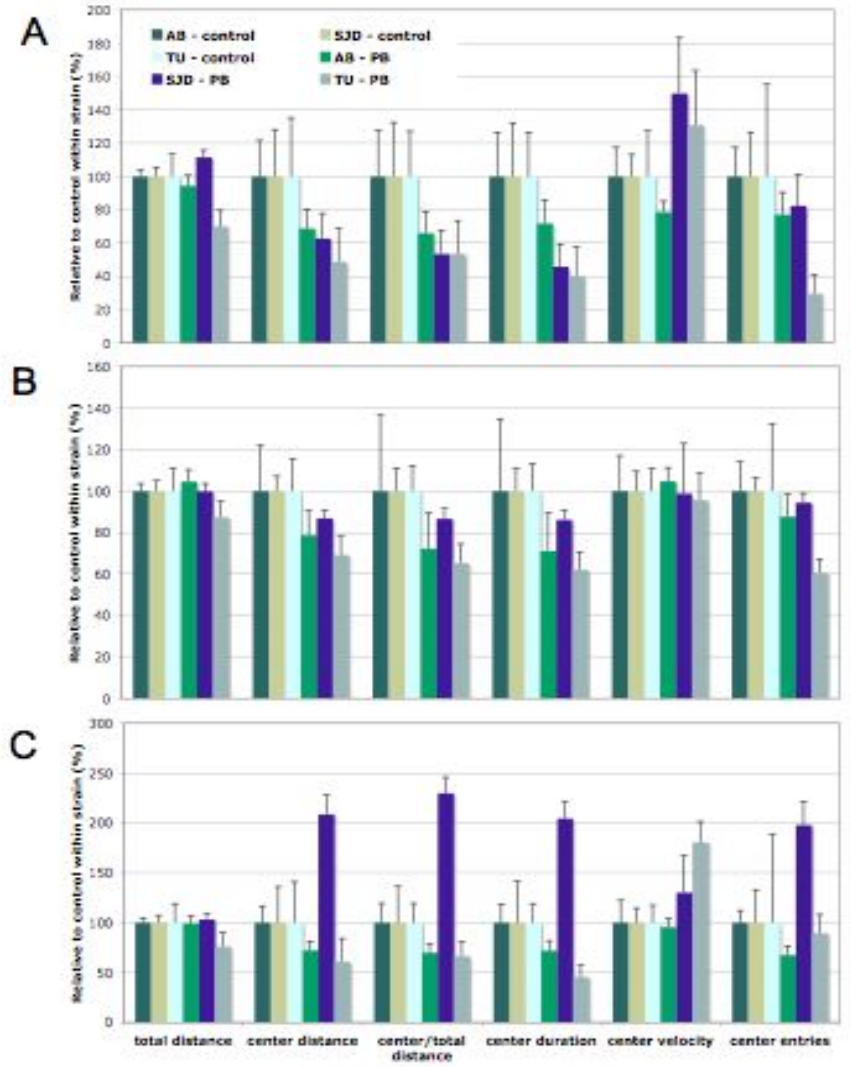


Figure 9. PB effects on motility and behavior by strain during (A) baseline light phase, (B) dark stimulus, and (C) post-stimulus light phase. Data are means (n = 23-24) ± s.e.

duration of movement in the center of the well was decreased by exposure to PB, suggesting that PB has anxiogenic effects in zebrafish larvae.

For the molecular characterization of the PB response in three strains of zebrafish, 54 samples (n=3 per treatment per strain) were used for mRNA-seq. RNA samples were prepared into libraries for mRNA-seq using the Nextera protocol (*9*) and sequenced as paired-end 50-bp on an Illumina HiSeq. With upgrades to our analysis pipeline following our preliminary experiments, we completed our alignments to the Ensembl database using TopHat (*22*) and compared the aligned transcriptome to the reference genome and RNAs present in the Refseq, Ensembl, and UCSC mRNA databases for identification of changes in the transcript sequence - such as indels, single- and multiple-nucleotide changes using snpEff (*23*). From the alignments, HTseq was utilized to generate raw counts for Ensembl genes (ens67). The analysis and interpretation of RNA-seq data may vary by the methodology used, so we compared several of

the most commonly used statistical packages for determination of differential expression such as DESeq, edgeR, and SAMseq (*24-26*). For the questions of interest in these experiments, our analysis indicated that DESeq was the most appropriate tool because SAMseq cannot handle multivariate models or interactions and edgeR had greater variability in the estimates of dispersion than DESeq. Therefore, DESeq was used for variance stabilization and differential expression calculations for the count data from HTSeq. With a threshold for the false discovery rate of 0.05, we found 356, 10,416, and 87 changes in gene expression attributable to the effects of PB, strain and PB x strain interaction, respectively. We used the DAVID Functional Annotation Tool (*12, 13*) to characterize the enrichment of altered transcripts. This identified the terms associated with PB treatment as endopeptidase activity, aminoacyl-tRNA biosynthesis, actin, and negative regulation of apoptosis (p53 signaling pathway). There was significant enrichment for terms associated with iron ion binding, DNA-dependent regulation of transcription, ribonucleotide binding, and alpha-linolenic acid metabolism in the genes altered by the PB x strain interaction. The dose-response to PB was also assessed using the ORIOGEN tool with the variance stabilized data produced by DESeq. For this analysis, samples within each strain were binned based on the AChE activity for the sample: control, PB-treated with less than 20% inhibition, 20-50% inhibition, and greater than 50% AChE inhibition. Using ORIOGEN to study the dose-response using this grouping identified 24 transcripts with a decreasing profile and 16 transcripts that were upregulated with increasing degrees of inhibition of AChE. The DAVID Functional Analysis Tool is generally not appropriate for gene lists this small; however, the Integrative Multi-species Prediction (IMP) tool (*27, 28*) was developed specifically for gene sets of this size and was used to search for enrichments of biological processes predicted to be involved with these genes. The upregulated genes were enriched for antigen processing and t-cell mediated cytotoxicity, whereas the downregulated genes were enriched for neuron synaptic transmission, positive regulation of G protein coupled receptor protein signaling pathway, circadian sleep wake cycle, regulation of renal sodium excretion, and elevation of cytosolic calcium ion concentration. Enrichment of biological processes for genes most downregulated at or below therapeutic levels of AChE inhibition also included dendrite guidance, visceral motor neuron differentiation, and muscle cell fate; upregulated genes were enriched for carbohydrate transport, peptidyl serine phosphorylation, long term memory, microtubule anchoring, long term potentiation, antibacterial humoral response, and regulation of microtubule polymerization.

Due to the large number of genes identified as statistically different between strains, subsequent analyses were undertaken within each strain to identify dose-dependent effects and those effects specifically associated with treatments near the clinically relevant range of ~20-50% inhibition of AChE activity (*1*). Similar to the results for the motility assay, there was the least significant response in the SJD strain, whereas analysis of the TU and AB strains produced larger sets of differentially expressed genes (**Figure 10**). Expression of 87 genes was altered in the SJD strain, with enrichment for peptidases. Exposure to PB altered expression of 235 transcripts in AB larval zebrafish, with enrichment for dynamin, organic acid biosynthetic process, oxidation-reduction, proteasome, and interferon regulatory factor. In the TU strain there was enrichment for response lectin/sugar binding, apoptosis, and response to bacterium in the 316 differentially regulated transcripts. The greatest degree of overlap in differentially expressed genes was found between the AB and TU strains (**Figure 10**) and within those 70 genes, there was overrepresentation within the interferon regulatory factors and the p53 signaling pathway. The mRNA-seq data from this experiment confirmed the findings of the preliminary studies with enrichments for both neurological function and calcium signaling, as well as for cytoskeletal structures, regulation of protein digestion and degradation, and regulation of cellular homeostasis, particularly redox and iron ion homeostasis.

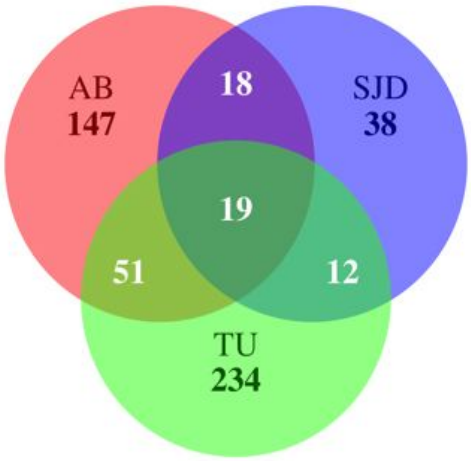


Figure 10. Overlap in genes differentially expressed in response to PB in larval zebrafish of three strains.

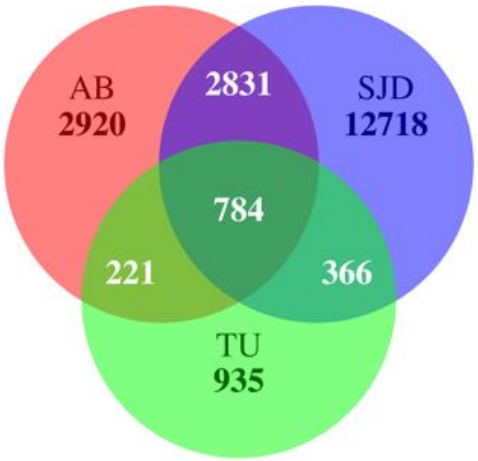


Figure 11. Overlap in SNPs identified by snpEff from RNA-seq data from three strains of zebrafish.

We have also begun exploring the data provided by the output of SnpEff (21), a SNP discovery and annotation tool. Using these algorithms, we identified 14,018 nonsynonymous nucleotide changes that might alter protein function. Of these SNPs, the fewest SNPs were observed in the TU strain and the greatest number of SNPs was found in the SJD strain (**Figure 11)**. The relative number of SNPs observed and extent of overlap between strains for differential expression and SNPs is consistent with our expectations because the TU strain was the strain used for the reference genome and the AB and TU strains are more genetically similar than the SJD strain (*29*). It is notable that there is little overlap in the PB-dose responsive genes between strains with over 70% of PB-responsive genes being unique to one strain. Although there is a greater degree of overlap in SNPs, no less than 40% of SNPs identified by strain are unique for that strain. Taken together with the motility data, this provides an excellent opportunity to understand genotype-driven strain-specific differences in gene expression, generally and specifically in response to PB exposure. The Fisher's exact test found that there was not a greater than expected number of SNPs within the differentially expressed genes within any of the strains. However, we anticipate that we will use the Gene Set Association Analysis-SNP (GSAA-SNP) software (*30*) to further investigate possible relationships between the SNPs identified within each strain and the differentially expressed gene data sets. This work could be translated into a greater ability to predict whether there are additional human populations that might be more susceptible to the effects of PB and whether the response to PB varies by genotypic differences at other loci beyond those for detoxification enzymes, such as butyrylcholinesterase and paraoxonase.

Effects of stress on the response to pyridostigmine bromide

In addition to exposure to PB, soldiers of the Gulf War of 1991 were also exposed to a wide variety of additional stressors. Given evidence for crosstalk between the acetylcholine and stress signaling pathway via a number of molecular mechanisms (*6, 31-33*), we sought to identify the effects of PB, with or without concurrent stress, in larval zebrafish of the AB strain. We conducted a study with the aim of understanding the effects of PB exposure and stress, alone or in combination, in larval zebrafish. Pooled groups of AB zebrafish larvae (n=20 larvae) were randomly assigned to one of four treatment groups: control, PB, stress, or PB+stress (n=24 per group). PB-treated fish were kept in 0.5 mmol PB/L for 72 hours from 4-7 days post-

Table 1. The effects of PB on acetylcholinestrase activities in larval zebrafish were not affected by stress. Data are means (n = 5) ± s.e.

Treatment	Acetylcholinesterase activity (% control)
control	100.0 ± 3.0
stress	96.6 ± 4.0
PB	56.0 ± 2.6 *
PB+stress	54.2 ± 3.2 *

*indicates significant difference from control (p<0.05)

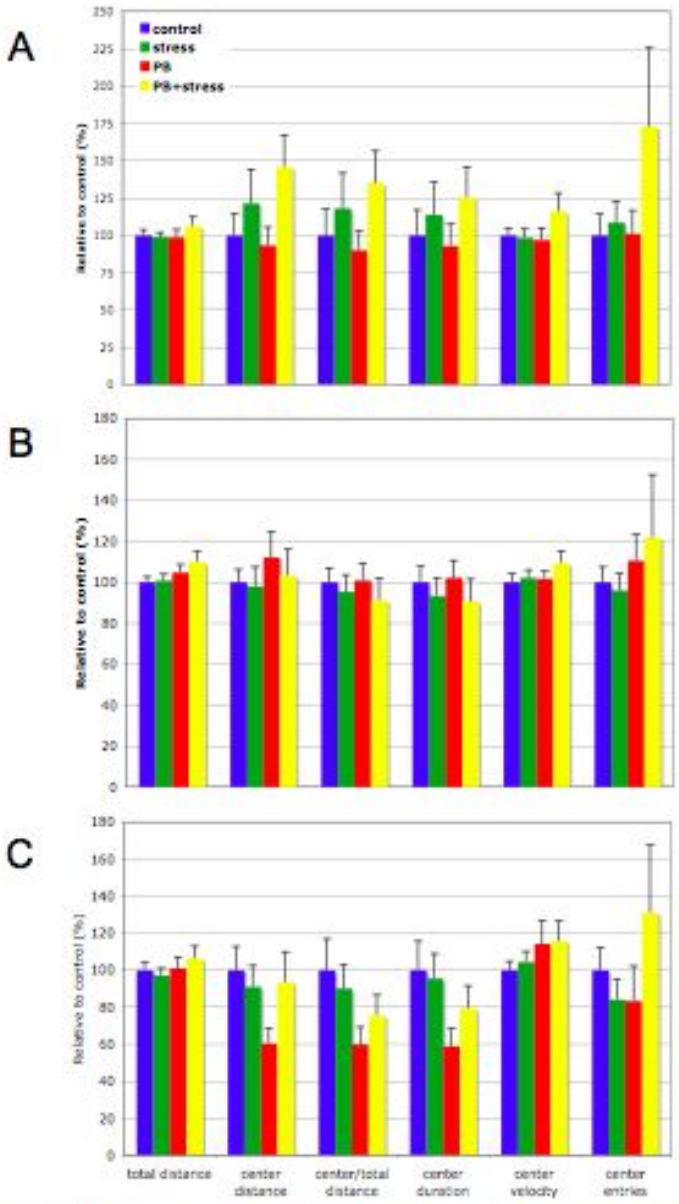


Figure 12. Motility and place preference are altered by PB and stress in larval zebrafish during (A) baseline light phase, (B) dark stimulus, and (C) post-stimulus light phase. Data are means (n = 31-36) ± s.e.

fertilization. To induce a stressful condition, larvae were swirled for 1 min twice per day starting at 4 dpf (*34*). Samples were collected for protein for enzymatic assays and for nucleic acid extraction at 7 dpf as described for the previous larval experiments, motility and behavior assays were also performed as described in **Figure 7**. Acetylcholinesterase activity was inhibited to within the clinically relevant range of 20-50% by PB exposure, whereas stressful conditions had no effect on acetylcholinesterase activity (**Table 1**).

A critical component of our research into the effect of PB is to determine whether the drug produces a phenotype that is comparable to the symptoms of Gulf War Illness. In this experiment, PB-treated larvae were most affected post-stimulus, with a lower percent of total distance traveled in the center and a lower duration of time spent in the center after the changing of light conditions, suggesting that PB-treated fish were more anxious (**Figure 12**). Conversely, during the dark phase stress-treated larvae tended to travel a greater distance in total and also a greater proportion of that distance tended to be in the center of the well, suggesting that inhibitions were decreased in stressed fish. When the data was binned by velocity, there were more high velocity movements in alarm pheromone-exposed fish during the dark phase, suggesting more erratic movement patterns induced by stressful conditions.

Nucleic acids were isolated from 20 samples (n=5 per group). We sequenced the RNAseq libraries as 50-bp paired end reads on an Illumina HiSeq, producing 66-73 million reads aligning to the zebrafish transcriptome for each sample. Using our analysis

pipeline as described for the natural genetic variation study we identified 151 genes differentially expressed in response to PB treatment, 358 genes differentially expressed under stressful conditions, and 57 genes for which there was an interaction between PB treatment and stress. Functional annotation clustering using the DAVID analysis tool revealed that within genes downregulated by PB there was overrepresentation of annexin, phospholipid binding, calcium binding, EF-hand motif, and actin, and within genes upregulated by PB there was enrichment for sequence-specific DNA binding. Transcripts altered by exposure to stress were enriched for the respiratory chain, ribosome, response to wounding, gelsolin and fibrinogen, interferon regulatory factor, thick filaments, cell-cell binding and calcium/phospholipids-binding and annexin. An interaction between PB and stress was associated with genes for extracellular regions, specifically extracellular matrix proteins. There was an overlap of 77 genes that were dysregulated by PB and stress, with no interaction; response to bacterium and calcium signaling were significantly enriched in these genes. These results confirm the effects of PB on genes associated with calcium signaling and cytoskeletal structures. Furthermore, we observed an interaction between PB and stress on genes for extracellular matrix proteins, which are also important for the determination of cell structure and function.

The studies of PB and stress exposures in larval zebrafish were useful for identifying large systemic changes in gene expression which might be relevant to Gulf War Illness, a disease that is characterized by a wide range of symptoms affecting many organ systems throughout the body. Although larval zebrafish have been shown to often be a good indicator of the toxicological response in adult fish, we aimed to further explore the effects PB and stress on specific tissues by also utilizing adult fish for our experiments. We developed a study to assess both the immediate and long-term effects of PB exposure, with and without co-exposure to stressful conditions. To this end, adult male zebrafish (AB strain, 4-12 months of age) were randomly assigned to tanks to receive control, PB, stress, or PB+stress treatments. PB-treated fish were kept in a 5 mmol/L PB solution and fish were stressed by 30 min exposure to alarm pheromone (*35*). A visual barrier was placed between tanks during stress treatment to preclude alterations in behavior based on movement in neighboring tanks. A 60% change of system water or PB solution was performed at the conclusion of the 30-minute stress treatment. System water served as the control for both PB and stress treatments. The fish in the experiments were raised on our recirculating system, acclimated to static tanks for two days and received experimental treatments for three days. Following the acclimation and treatment period, fish from half of the tanks (group A) were euthanized for sample collection and the remaining fish (group B) were used for motility assays to assess neurobehavioral phenotypes. (**Figure 13**).

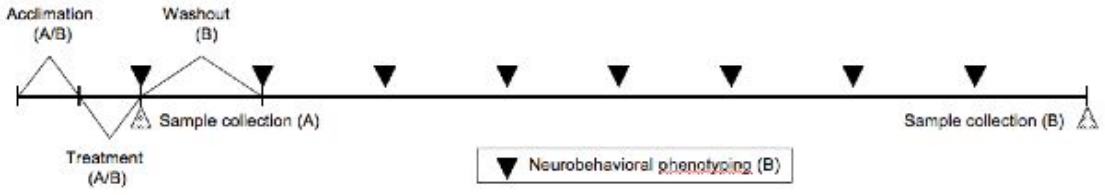


Figure 13. Experimental design for study of effects of PB and/or stress in adult zebrafish. Capital letters indicate fish used for sample collection at 0-wk (A) or 8-wk (B) post-treatment.

The sample collection included excising a portion of skeletal muscle and the brain for the isolation of enzymatically-active protein and nucleic acids from each tissue, as well as whole body samples for determination of cortisol levels. For the motility analysis, fish were moved

individually from the group treatment tank into 100 mL of system water and allowed to acclimate for 15 min on the counter, followed by 5 min of acclimation in the ZebraBox. After the acclimation period, the fish was subjected to the experimental protocol of light:dark disruption as described for larval zebrafish. Fish used for motility assays were moved to static tanks containing system water for a 7-day washout period before returning to the system. These fish were assessed weekly for sustained changes in locomotion or behavior, with sample collection at 8 weeks after the treatment period (**Figure 13**). For the motility assay, this experimental series was repeated twice: experimental series 1 consisted of four replications of the protocol, experimental series 2 consisted of three replications of the protocol, but with motility analysis only at 0, 4 and 8 weeks post-treatment to minimize the possibility of habituation being a confounding factor in the analysis. In both series, animals were between 4 and 12 months of age and one replication in each experimental series was conducted with female fish. Fish from experimental series 2 were euthanized for sample collection at 12 weeks post-treatment; these samples have been stored at -80°C and may be used for validation or follow up studies.

Skeletal muscle AChE activity was altered immediately after treatment by an interaction between PB and stress ($p = 0.058$), with stress attenuating the AChE inhibiting effect of PB (**Figure 14,** control vs. PB pair wise t-test $p = 0.03$). There were no changes observed in muscle AChE activity at 8-wk post-treatment or in brain AChE activities at either time point. For experimental series 1, immediately following the cessation of treatment, fish treated with PB traveled a shorter distance during all phases of the experiment, however a greater amount of time was spent in the center of the well and specifically during the dark period there were more entries to the center and a greater distance traveled in the center as well (**Figure 15**). Stress decreased the total distance traveled and lowered the average velocity of movement overall and in the center of the well during the dark period, an effect that was opposite of the observed in larval zebrafish and may reflect differences in the methodologies used to induce the stressful condition or an age-related difference in the stress response. Replication of the experiment during experimental series two was designed to assess neurobehavioral characteristics only immediately after treatment and at 4 and 8 weeks post-treatment to minimize the possibility of habituation occurring with increasing repetitions of the protocol and thereby confounding the results. RNA-seq libraries were prepared and sequenced for brain and skeletal muscle collected immediately after the exposure period (n = 4 per treatment group) and at 8-wk post-treatment. The increase in distance traveled in center as percent total at all phases was also replicated in experimental series two. The decrease in total distance and increase in duration spent in the center during baseline was also replicated. Stress also resulted in a significantly decreased center velocity and there tended to be decreased total distance traveled in dark phase in fish from experimental series two. The data from experimental series one weeks one through seven was analyzed using a linear regression model taking into account the main effects and interactions of PB, stress, and time post-treatment. In this analysis, there remained significant effects of PB treatment, stress, and several instances in which a PB x stress interaction was observed (**Table 2**). PB treatment resulted in a significant increase in the duration and distance traveled in center as percent total during the first light period. Exposure to alarm pheromone resulted in an increase in the

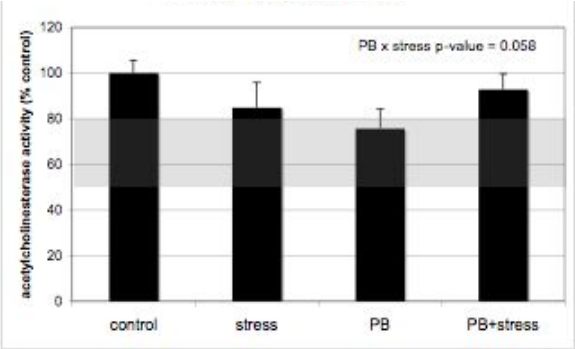


Figure 14. Acetylcholinesterase activity was inhibited to within therapeutic levels in the PB-treated muscle at 0-d post-treatment. Data are means (n = 4) ± s.e.

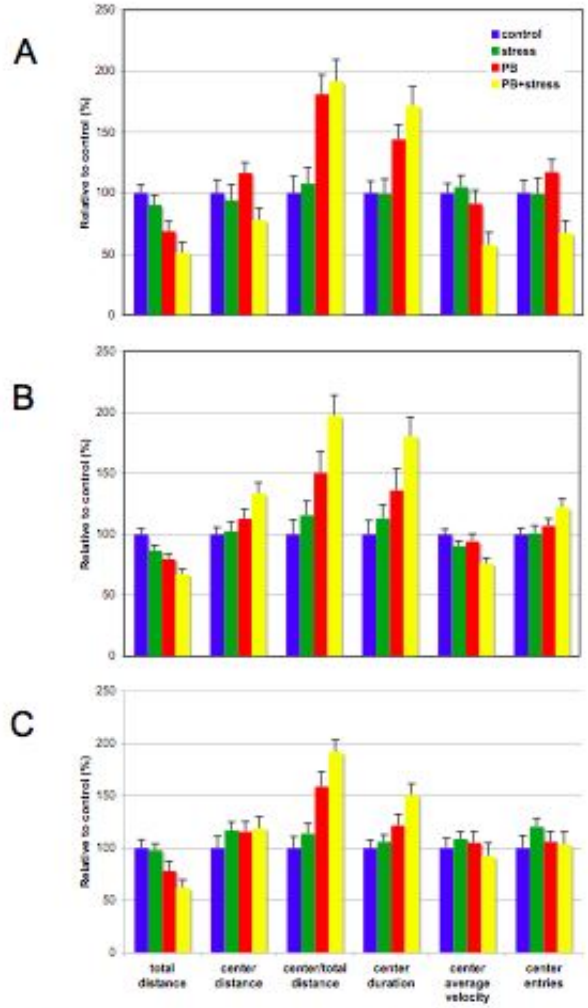


Figure 15. Locomotion and behavior are altered in adult zebrafish following 72-h PB and/or alarm pheromone exposure during (A) baseline light phase, (B) dark stimulus, and (C) post-stimulus light phase. . Data shown are from experimental series 1, data are means (n = 16) ± s.e.

Table 2. An interaction between PB and stress modulates motility and behavior in adult zebrafish in the weeks after exposure.

	Total distance (mm)	Center distance (mm)	Center/total distance	Center duration (s)	Center average velocity (mm/s)	Center entries (count)
Baseline light phase						
control	14977 ± 1139***	3499 ± 268	0.27 ± 0.03*	103.6 ± 10.9*	39.3 ± 3.3***	94.4 ± 5.7
stress	13064 ± 1208	3786 ± 474	0.31 ± 0.05	114.3 ± 13.9	33.6 ± 3.0	102.4 ± 11.8
PB	14381 ± 899	3883 ± 344	0.31 ± 0.04	108.7 ± 12.1	38.4 ± 2.4	110.3 ± 9.1
PB+stress	11783 ± 1104	3872 ± 431	0.38 ± 0.04	121.8 ± 13.4	34.9 ± 2.9	111.5 ± 12.5
Stimulus dark phase						
control	14646 ± 621***	1920 ± 120***	0.14 ± 0.01**	45.6 ± 5.2***	46.9 ± 2.4***	62.0 ± 3.5***
stress	12622 ± 694	1726 ± 125	0.15 ± 0.01	47.6 ± 4.8	39.6 ± 2.4	58.1 ± 4.3
PB	13003 ± 630	1659 ± 107	0.14 ± 0.01	44.3 ± 5.0	42.0 ± 2.5	55.8 ± 3.8
PB+stress	11510 ± 611	2387 ± 194	0.25 ± 0.04	76.7 ± 11.6	37.7 ± 2.2	76.0 ± 4.8
Post-stimulus light phase						
control	13756 ± 893***	4618 ± 419	0.38 ± 0.04	139.6 ± 12.3	35.7 ± 2.4***	116.0 ± 10.8
stress	12653 ± 1230	4641 ± 504	0.43 ± 0.05	143.3 ± 12.8	34.2 ± 3.0	116.7 ± 12.3
PB	13211 ± 867	4016 ± 348	0.34 ± 0.04	122.4 ± 11.1	34.6 ± 2.0	111.0 ± 8.9
PB+stress	12632 ± 967	4147 ± 272	0.39 ± 0.04	133.6 ± 13.0	36.0 ± 2.7	114.0 ± 8.0

* $p < 0.05$ for main effect of PB
** $p < 0.05$ for main effect of stress
*** $p < 0.05$ for PB x stress interaction

percentage of distance traveled in the center during the dark phase. In both experimental series, in the weeks after treatment, in the dark phase there were divergent behavioral responses to PB based on the presence or absence of stressful exposure. PB+stress increased entries to center as well as center distance and duration and decreased total distance and center average velocity, whereas the opposite effect was observed in animals only treated with PB. We have found in these two experimental series that PB and stress exposures alter motility and behavioral phenotypes in adult zebrafish; the specific effects of PB treatment included decreased total movement distance and duration immediately following treatment, whereas PB treatment and/or PB+stress treatment produced a more complex neurobehavioral phenotypes for weeks after treatment as evidenced by altered patterns in movement in the center of the field.

Libraries for mRNA-seq (n = 4 per treatment, male fish) were sequenced as 50-bp paired-end reads on an Illumina HiSeq, producing at least 20 million reads for each sample. Reads were aligned to the transcriptome using Tophat, raw counts were determined using HTSeq-count, and differential expression of genes was assessed using DESeq with a false discovery rate (fdr) cutoff of < 0.05. In both tissues and at both 0-d and 8-wk timepoints, we identified sets of differentially expressed genes in response to PB, stress, and a PB x stress interaction (**Table 3**).

Muscle	0-wk post-treatment	8-wk post-treatment
PB	306	303
stress	80	67
PB x stress	39	879
Brain	0-wk post-treatment	8-wk post-treatment
PB	427	131
stress	273	204
PB x stress	399	323

Table 3. Differentially expressed genes in the brain or muscle of adult zebrafish after treatment with PB and/or alarm pheromone.

A targeted inspection of the data revealed that several genes related to acetylcholine signaling were dysregulated. Subunits of the nicotinic acetylcholine receptor were downregulated in both the brain and muscle immediately after the treatment period. We also observed downregulation of choline acetyltransferase in the muscle and of several synaptic proteins in the brain at this time point. Many of the same or related genes were differentially expressed at 8-wk post-treatment, although there was an interaction between PB and stress at that time point and the pattern of expression among treatment groups was not consistent.

There was minimal overlap between the specific genes in each differentially expressed gene sets, however, when the DAVID Functional Analysis Tools were used to identify functional enrichment there were several instances of overlap in the functional enrichment categories for PB differentially expressed genes in both tissues at the 0-wk time points in broad functional categories such as regulation of transcription, iron ion binding, and oxidation reduction (**Table 4**). Further analysis of the genes found in these categories may yield novel insights into the early systemic pathogenesis of Gulf War Illness. However, there were also instances of functional overlaps at both 0-d and 8-wk post-treatment within a particular tissue. In muscle, these terms were related to cytoskeletal structure, specifically intermediate filament, gap junction and connexins for PB-responsive genes. In brain, the extracellular matrix proteins were overrepresented at both time points. In addition to those genes perturbed by PB treatment,

Table 4. Overlapping terms for DE genes significant for an effect of pyridostigmine bromide in adult zebrafish tissues. Data are p-values with FDR rate correction.

		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
GOTERM_BP_FAT	GO:0055114~oxidation reduction	49.58		**0.11**	
GOTERM_MF_FAT	GO:0005506~iron ion binding	61.99		**0.08**	
SP_PIR_KEYWORDS	heme	66.75		**1.28**	
SP_PIR_KEYWORDS	iron	64.78		**0.29**	
GOTERM_MF_FAT	GO:0003700~transcription factor activity	**0.01**		55.62	
SP_PIR_KEYWORDS	developmental protein	**0.05**		52.13	
SP_PIR_KEYWORDS	dna-binding	**<0.01**		50.82	
GOTERM_CC_FAT	GO:0005856~cytoskeleton			16.58	**3.44**
GOTERM_CC_FAT	GO:0005882~intermediate filament			**0.02**	53.77
GOTERM_CC_FAT	GO:0005921~gap junction			53.18	**0.36**
GOTERM_CC_FAT	GO:0005922~connexon complex			47.50	**0.25**
GOTERM_CC_FAT	GO:0044430~cytoskeletal part			5.33	**4.82**
GOTERM_CC_FAT	GO:0045111~intermediate filament cytoskeleton			**0.02**	53.77
INTERPRO	IPR000500:Connexins			54.41	**0.46**
INTERPRO	IPR001664:Intermediate filament protein			**0.02**	68.38
INTERPRO	IPR013092:Connexin, N-terminal			52.33	**0.41**
INTERPRO	IPR016044:Filament			**0.02**	68.38
INTERPRO	IPR017990:Connexin, conserved site			50.20	**0.36**
INTERPRO	IPR017991:Connexin region			54.41	**0.46**
INTERPRO	IPR019570:Gap junction protein, cysteine-rich domain			54.41	**0.46**
SMART	SM00037:CNX			29.28	**0.49**
SP_PIR_KEYWORDS	cell junction			49.38	**2.48**
SP_PIR_KEYWORDS	gap junction			45.89	**0.39**
SP_PIR_KEYWORDS	Intermediate filament			**0.01**	61.79
GOTERM_CC_FAT	GO:0005576~extracellular region	**3.92**	39.80		

Table 5. Overlapping terms for DE genes significant for PB x stress interaction. Data are p-values with FDR rate correction.

		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism	25.02	5.75	21.38	**0.11**
GOTERM_BP_FAT	GO:0006814~sodium ion transport		63.65		**<0.01**
GOTERM_BP_FAT	GO:0055085~transmembrane transport		24.06		**0.05**
GOTERM_CC_FAT	GO:0005882~intermediate filament		**0.01**		**<0.01**
GOTERM_CC_FAT	GO:0005886~plasma membrane		9.52		**<0.01**
GOTERM_CC_FAT	GO:0005911~cell-cell junction		**4.19**		16.31
GOTERM_CC_FAT	GO:0016327~apicolateral plasma membrane		**3.41**		7.72
GOTERM_CC_FAT	GO:0030054~cell junction		9.22		**0.11**
GOTERM_CC_FAT	GO:0043296~apical junction complex		**3.41**		7.72
GOTERM_CC_FAT	GO:0044430~cytoskeletal part		**0.83**		**0.94**
GOTERM_CC_FAT	GO:0044459~plasma membrane part		8.09		**<0.01**
GOTERM_CC_FAT	GO:0045111~intermediate filament cytoskeleton		**0.01**		**<0.01**
GOTERM_MF_FAT	GO:0005198~structural molecule activity		**0.32**		**0.15**
INTERPRO	IPR002957:Keratin, type I		**0.30**		**<0.01**
INTERPRO	IPR006187:Claudin		72.74		**0.32**
INTERPRO	IPR016044:Filament		**0.04**		**<0.01**
INTERPRO	IPR018039:Intermediate filament protein, conserved site		14.12		**1.34**
PIR_SUPERFAMILY	PIRSF002282:cytoskeletal keratin		52.82		**3.69**
SP_PIR_KEYWORDS	Intermediate filament		**0.04**		**<0.01**
SP_PIR_KEYWORDS	keratin		**<0.01**		26.37
GOTERM_MF_FAT	GO:0005506~iron ion binding		64.82	62.72	**1.29**
INTERPRO	IPR001128:Cytochrome P450		64.67	62.73	**3.98**
COG_ONTOLOGY	Signal transduction mechanisms / Cytoskeleton / Cell division and chromosome partitioning / General function prediction only	9.79	**0.13**		52.26
INTERPRO	IPR002048:Calcium-binding EF-hand	**1.71**			49.05
INTERPRO	IPR018248:EF hand	**4.36**	9.78		14.39
SMART	SM00054:EFh	**3.49**			23.44
SP_PIR_KEYWORDS	calcium	**2.64**	**1.99**		41.55
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	14.50	**0.04**		
GOTERM_BP_FAT	GO:0007600~sensory perception	**0.01**	**<0.01**		
GOTERM_BP_FAT	GO:0007601~visual perception	**0.04**	**0.14**		
GOTERM_BP_FAT	GO:0045449~regulation of transcription	33.52	**3.91**		
GOTERM_BP_FAT	GO:0050877~neurological system process	**0.02**	**0.03**		
GOTERM_BP_FAT	GO:0050890~cognition	**0.01**	**<0.01**		
GOTERM_BP_FAT	GO:0050953~sensory perception of light stimulus	**0.04**	**0.14**		
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	16.25	**0.04**		
GOTERM_MF_FAT	GO:0003700~transcription factor activity	**0.87**	**<0.01**		
GOTERM_MF_FAT	GO:0004112~cyclic-nucleotide phosphodiesterase activity	**0.09**	**0.05**		
GOTERM_MF_FAT	GO:0004114~3',5'-cyclic-nucleotide phosphodiesterase activity	**0.09**	**0.05**		
GOTERM_MF_FAT	GO:0005179~hormone activity	**0.28**	**9.24**		
GOTERM_MF_FAT	GO:0008081~phosphoric diester hydrolase activity	**5.52**	**3.41**		
GOTERM_MF_FAT	GO:0019002~GMP binding	57.50	**1.58**		
GOTERM_MF_FAT	GO:0030528~transcription regulator activity	**2.01**	**0.54**		
GOTERM_MF_FAT	GO:0030551~cyclic nucleotide binding	65.69	**2.60**		
GOTERM_MF_FAT	GO:0030553~cGMP binding	57.50	**1.58**		
GOTERM_MF_FAT	GO:0043565~sequence-specific DNA binding	**1.73**	**<0.01**		
INTERPRO	IPR000698:Arrestin	76.92	**4.72**		
INTERPRO	IPR001356:Homeobox	**<0.01**	**<0.01**		
INTERPRO	IPR008952:Retinal cGMP phosphodiesterase, gamma subunit	56.73	**1.41**		
INTERPRO	IPR012287:Homeodomain-related	**<0.01**	**<0.01**		
INTERPRO	IPR014753:Arrestin, N-terminal	76.92	**4.73**		
INTERPRO	IPR017970:Homeobox, conserved site	**0.01**	**<0.01**		
PIR_SUPERFAMILY	PIRSF000969:3',5'-cyclic-GMP phosphodiesterase, gamma subunit	38.84	**1.00**		
PIR_SUPERFAMILY	PIRSF000969:35-cGMP_Pdiase_g	48.09	**1.97**		
SMART	SM00389:HOX	**0.02**	**<0.01**		
SP_PIR_KEYWORDS	developmental protein	39.27	**0.02**		
SP_PIR_KEYWORDS	DNA binding	11.83	**1.13**		
SP_PIR_KEYWORDS	dna-binding	**0.01**	**<0.01**		
SP_PIR_KEYWORDS	Homeobox	**<0.01**	**<0.01**		
SP_PIR_KEYWORDS	nucleus	18.60	**3.57**		
UP_SEQ_FEATURE	DNA-binding region:Homeobox	**1.38**	**<0.01**		

there was enrichment for genes related to sensory perception, hormone activity, and regulation of transcription in expression altered by a PB x stress interaction (**Table 5**).

Consistent with reports of crosstalk and similar molecular perturbation as a result of PB and stressful exposures, the steroid biosynthesis pathway was common to several of our differentially-expressed gene lists. In the muscle, steroid and lipid biosynthetic processes were downregulated by PB treatment at 0-d post-treatment. Steroid biosynthesis was also stronglydownregulated by the stress treatment in both the brain and muscle at the end of the 72-hr treatment period. However, a PB x stress interaction was observed for this process in the muscle at 8-wk post-treatment, with gene expression levels being significantly greater in animals treated with alarm pheromone only compared to the other groups.

There was enrichment for a number of biologically interesting term categories in the PB x stress interaction gene sets from muscle at 8-wk post-treatment, such as voltage-gated ion channel activity, neurotransmitter transport, clathrin-coated vesicle, and cell junction (PB, stress, and PB x stress enrichment terms found in only one tissue and time point found in **Supporting Data**). Enrichment was also observed for the cell junction in the muscle at 8-wk post-treatment for the genes that were downregulated in response to PB treatment. At 8-wk post-treatment, we identified several functional categories that were enriched in differentially expressed genes from both brain and muscle in response to PB treatment or a PB x stress interaction, specifically, the INTERPRO categories for claudin and low density lipoprotein-receptor class A proteins, as well as the tight junction GO term and KEGG pathway for cell adhesion molecules.

These functional categories that have been found to be over-represented in our differentially-expressed gene sets have plausible biological connections to the neuromuscular symptoms of Gulf War Illness and warrant further validation. These insights also offer promise for the development of therapies for the treatment of Gulf War Illness.

Technical objective 3: assessment of off-target effects by genetic and chemical means

To complement our studies of PB in the presence or absence of stressful conditions in AB zebrafish larvae with a genetic approach, we have also performed experiments to study the response to PB and/or stress in the ache^{tm205} and ache^{tf222a} mutant strains. This line of inquiry may suggest whether the effects of PB are intrinsic to changes in acetylcholinesterase activity levels or whether they may be attributed primarily to off-target effects. Pooled groups (n=20) of ache^{tm205} or ache^{tf222a} larvae from clutches produced by either homozyogous wild-type or heterzyogous carriers were randomized to receive stress treatments with the experimental protocol as described for AB larvae. The background strain for both mutants is the AB strain, however the *ache^{tf222a}* genotype has a point mutation adjacent to the catalytic site of AChE, whereas the *ache^{tm205}* mutation creates a premature stop codon, resulting in a protein lacking both the catalytic site and the C-terminal domain (*36*). Both mutations were reported to abolish AChE enzymatic activity. Given that both of the *ache* mutations are lethal at 5 dpf, two-thirds of the larvae from a cross of two heterozygous adults would be expected to be two-thirds heterozygous carriers of the mutation. Assuming a complete lack of activity from the mutant allele, no increase in the expression of AChE and complete penetrance, it would be expected that AChE activity would be decreased by ~33% in samples from pooled heterozygous cross offspring samples and this is what we have observed in the *ache^{tm205}* strain (**Table 6**). AChE

activity was significantly decreased to 25% in the *ache*^{tf222a} heterozygous larvae; there was no effect of stress observed in any strain.

Table 6. PB exposure or heterozygosity for *ache* mutant allele decreased acetylcholinesterase activity in larval zebrafish. Data are means ± s.e.

Strain	Inhibition relative to control (mean ± s.e.)	p-value
AB	40.1 ± 3.4%	< 0.01
ache^{tm205}	34.0 ± 9.9%	0.038
ache^{tf222a}	25.4 ± 2.9%	0.022

To further characterize which mutant strain produces a phenotype most similar to PB-treated AB larvae, we also conducted the motility/neurobehavioral assay (as in **Figure 7**) and genotyped each larvae to identify heterozygous carriers. When compared to the strongest phenotype observed in PB-treated AB larvae, i.e. decreased distance and duration in the center of the well during the post-stimulus period, we found that there was a significant decrease in the distance traveled and a trend for decreased duration (p = 0.084) in *ache*$^{tm205-/+}$ larvae compared to wild type larvae, whereas no differences were observed in *ache*$^{tf222a-/+}$ larvae (**Figure 16**). This is consistent with the motility data from the initial identification and characterization of the abnormal movement in the *ache* mutants that found most pronounced phenotype in *ache*^{tm205} homozygotes (*37*). This data suggests that the *ache*^{tm205} mutation produces phenotypes that are most similar to the phenotypes resulting from PB treatment. By comparing the chemical treatment to the genetic model at the molecular level, we could determine whether the effects of PB are through its primary mechanism of acetylcholinesterase inhibition or whether off-target effects that could contribute to the development of Gulf War Illness.

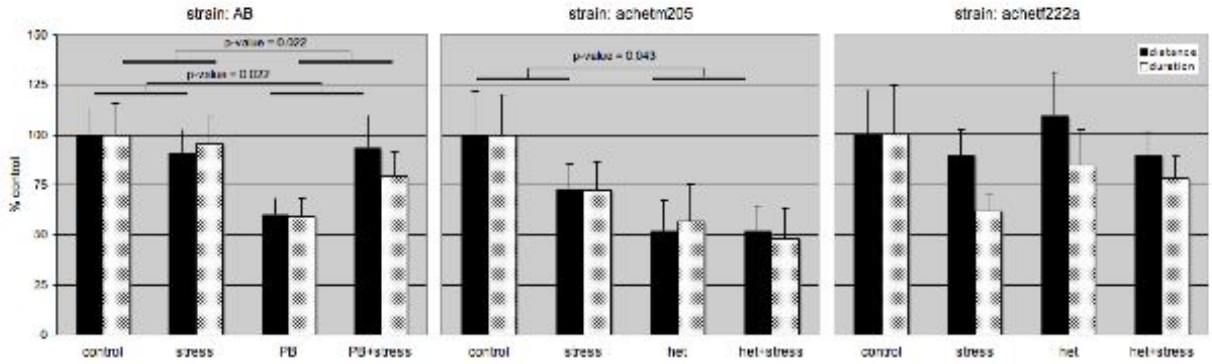


Figure 16. AB larvae treated with PB and heterozygous *ache*^{tm205} larvae spent traveled less distance and/or spent less time in the center of the well during the post-stimulus period in the motility analysis, whereas no difference in behavior were noted *ache*^{tf222a}. Data are means (n = 14-36) ± s.e.

Given the possibility that off-target effects of PB may be related to the symptoms of Gulf War Illness, it may be desirable to consider other AChE inhibitors (AChEIs) as potential alternatives for pretreatment during the threat of nerve agent exposure. With this in mind, we chose to study the response to galantamine (GAL, brand name Razadyne), rivastigmine (RIV, brand name Exelon), and donepezil (brand name Aricept). These chemicals are all water-soluble AChEIs that are currently approved by the FDA for the treatment of Alzheimer's disease and have been

studied for their use against nerve agents. Larvae were treated with 0.1 mM acetylcholinesterase inhibitor solution or water for 72 hrs. Exposure to the donepezil solution resulted in 100% mortality of larvae within 24-h after administration, the experiment was continued with the remaining treatment groups. At the end of the treatment period, protein and RNA samples were prepared, AChE activity was quantified, and mRNA-seq libraries were prepared and sequencing data analyzed as described for the larval studies under objective one. The mRNA-seq libraries (n = 3 per treatment) were sequenced as single-end 36-bp on an Illumina GAIIx, producing ~9-30 million reads alignable to Zv9 exons per sample.

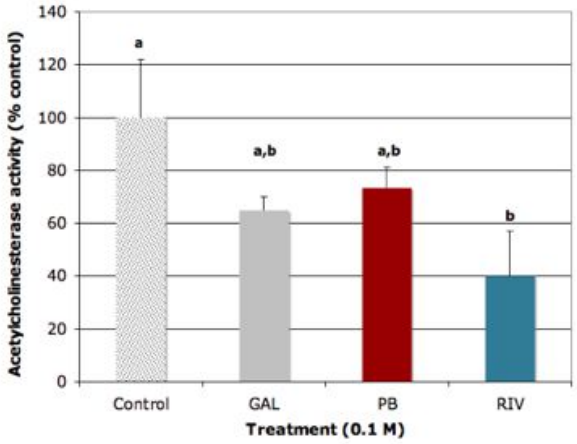


Figure 17. Acetylcholinesterase activity in larval zebrafish was inhibited by 72-h treatment with acetylcholinesterase inhibitors galantamine (GAL), pyridostigmine bromide (PB), and rivastigmine (RIV). Data are means ± s.e.

The treatment of larval zebrafish with AChEIs was successful in inhibiting AChE activity (**Figure 17**). Acetylcholinesterase was inhibited to approximately the range of clinical relevance for GAL and PB treatments. Rivastigmine treatment produced 60% inhibition of AChE activity, which was significantly different from the control. Similar to the pattern for AChE activities, the greatest number of differentially expressed transcripts relative to control was found in response to RIV treatment, with both GAL and PB also inducing alterations in gene expression, but to a lesser extent (**Figure 18**). Genes shared by GAL and RIV were strongly enriched for heat shock proteins, glutathione S-transferase, short-chain dehydrogenase/reductase, carbonic anhydrase, scavenger receptor activity, and extracellular matrix. Genes differentially expressed by PB and GAL were enriched several terms we have reported for the PB response in our other experiments, such as neurological system processes, synapse, nucleotide binding, and ion transmembrane transport. Genes unique to the PB response in this data set were enriched for terms associated with sensory perception, specifically retinal proteins. These results are suggestive that there are few off-target effects of PB. However, there were a large number of genes unique to GAL and RIV exposure with significant enrichments. GAL genes were overrepresented for structural molecules of the ribosome and mitochondria, oxidative phosphorylation and cellular cation homeostasis, whereas RIV genes were enriched for beta/gamma crystallins, peptidases, intermediate filaments, EF hand, lipocalin, metabolism of xenobiotics by cytochrome P450, proteinase inhibitiors, carboxylesterases, and calreticulin/calnexin. Interestingly, the enrichments for the RIV unique DE gene list share some commonalities with those found in the early dose response study for

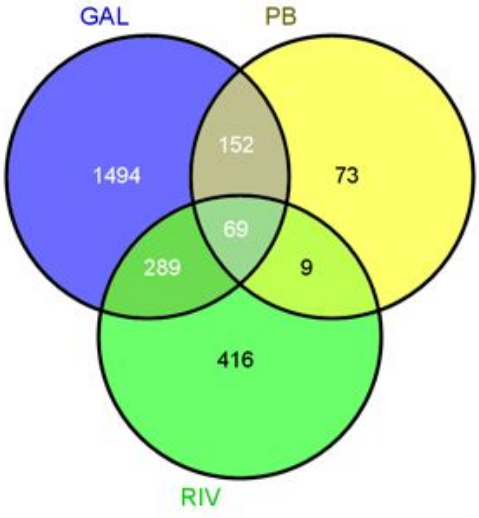


Figure 18. Treatment of larval zebrafish with acetylcholinesterase inhibitors results in differential gene expression. Numbers indicate count of differentially expressed transcripts (adjusted $p < 0.05$).

the largest dose of PB, which produced levels of AChE inhibition similar to that produced by RIV treatment. In sum, the effects of PB on gene expression appear to be a secondary consequence of AChE inhibition as evidenced by similar perturbation of gene expression in response to other AChEIs.

Technical objective 4: exposure of fish to chemical with screening for resistance

In preparation for a screening of the offspring of mutagenized fish for increased resistance to the effects of PB, we exposed larvae to suprapharmacological PB doses (0.01-0.5 mol/L) to determine the dosage at which PB exposure is lethal and characterized the resulting movement phenotypes at sublethal doses. AB larvae were placed individually in the wells of a 96-well plate and treated with 200 μL of PB solution (0.01, 0.025, 0.05, 0.1, 0.25 or 0.5 mol/L) with randomization by column (n=32 larvae per treatment). After 24 hours of treatment, one hour of tracking under normal light conditions was used to determine movement patterns and was used in conjunction with visual assessment to determine the number of larvae surviving. While 66% of larvae survived at 0.05 mol PB/L, only 1 fish survived the 0.1 mol PB/L dose and none of the fish exposed to either 0.25 or 0.5 mol/L were alive after 24 hours of treatment. As the dose of PB increased, the duration and distance of movement of surviving fish decreased. By tracking movement as well as measuring the survival, it would be possible to develop a more sensitive screen in which attenuation of the movement phenotype could also be utilized as an indicator of resistance to PB.

KEY RESEARCH ACCOMPLISHMENTS

Over the duration of this award we:

- Built an aquaculture facility with active breeding colonies of 5 strains of zebrafish.
- Completed dose-response and time-course studies of PB exposure in larval zebrafish.
- Studied the effect of natural variation on the response to PB in larval zebrafish by comparing phenotypes and dose-responsive gene expression patterns in three strains.
- Determined the effect of stress on motility and behavioral phenotypes, as well as gene expression changes in PB-treated larval and adult zebrafish.
- Utilized *ache* mutants as a genetic model and additional acetylcholinesterase inhibitors as a chemical model for studying the potential off-target effects of PB.
- Identified whole-body and tissue-specific perturbations of gene expression in response to PB treatment using larval and adult zebrafish respectively.

REPORTABLE OUTCOMES

A manuscript is in preparation, with submission expected by the end of 2013.

CONCLUSION

In summary, we have utilized motility/behavioral phenotyping and transcriptional profiling in larval and adult zebrafish to increase understanding of the effects of pyridostigmine bromide and factors of relevance to the combat situation in the 1991 Gulf War, such as stress and genetic variation. Our data indicate that PB exposure alters behavior in larval and adult zebrafish as indicated by altered place preference, although in larval zebrafish the response tended to be anxiogenic, whereas an anxiolytic effect was observed in adults. Our analysis of the molecular response to PB, as measured using mRNA-seq, repeatedly identified genes related to the synapse, calcium binding and signaling, cytoskeletal parts, and cell junctions as downregulated. Immediately after treatment with the drug, we also consistently observed perturbations in genes affecting oxidation-reduction reactions, regulation of transcription, lipid metabolic processes, and iron ion homeostasis. Similar categories of genes were differentially-expressed in larval zebrafish treated with the acetylcholinesterase inhibitors galantamine and rivastigmine, suggesting that many of these alterations of gene expression occur specifically due to the downstream effects of lowered acetylcholinesterase activity and that PB does not have broad off-target effects. Identification of these categories of differentially-expressed genes may offer potential targets for therapies for the treatment Gulf War Illness or aid in the prevention of the development of similar pathologies in future conflicts.

REFERENCES

1. Insititute of Medicine, *Gulf War and Health. Volume 1. Depleted Uranium, Pyridostigmine Bromide, Sarin, and Vaccines.* C. L. CE Fulco, HC Sox, Ed., (National Academy Press, Washington, D.C., 2000).
2. K. E. Lucas, P. C. Rowe, H. K. Armenian, Latency and exposure-health associations in Gulf War veterans with early fatigue onsets: a case-control study. *Ann Epidemiol* **17**, 799 (Oct, 2007).
3. L. Steele, A. Sastre, M. M. Gerkovich, M. R. Cook, Complex Factors in the Etiology of Gulf War Illness: Wartime Exposures and Risk Factors in Veteran Subgroups. *Environ Health Perspect*, (Sep 19, 2011).
4. I. Lamproglou *et al.*, Repeated stress in combination with pyridostigmine Part I: long-term behavioural consequences. *Behav Brain Res* **197**, 301 (Feb 11, 2009).
5. K. Husain, S. M. Somani, Persistent/delayed toxic effects of low-dose sarin and pyridostigmine under physical stress (exercise) in mice. *Indian J Physiol Pharmacol* **48**, 150 (Apr, 2004).
6. L. Barbier *et al.*, Repeated stress in combination with pyridostigmine Part II: changes in cerebral gene expression. *Behav Brain Res* **197**, 292 (Feb 11, 2009).
7. Y. Loewenstein-Lichtenstein *et al.*, Genetic predisposition to adverse consequences of anti-cholinesterases in 'atypical' BCHE carriers. *Nat Med* **1**, 1082 (Oct, 1995).
8. R. W. Haley, S. Billecke, B. N. La Du, Association of low PON1 type Q (type A) arylesterase activity with neurologic symptom complexes in Gulf War veterans. *Toxicol Appl Pharmacol* **157**, 227 (Jun 15, 1999).
9. J. Gertz *et al.*, Transposase mediated construction of RNA-seq libraries. *Genome Res* **22**, 134 (Jan, 2012).
10. B. Langmead, C. Trapnell, M. Pop, S. L. Salzberg, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10**, R25 (2009).

11. T. E. Reddy, J. Gertz, G. E. Crawford, M. J. Garabedian, R. M. Myers, The hypersensitive glucocorticoid response specifically regulates period 1 and expression of circadian genes. *Mol Cell Biol* **32**, 3756 (Sep, 2012).
12. W. Huang da, B. T. Sherman, R. A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44 (2009).
13. W. Huang da, B. T. Sherman, R. A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**, 1 (Jan, 2009).
14. S. J. Simmons, S. D. Peddada, Order-restricted inference for ordered gene expression (ORIOGEN) data under heteroscedastic variances. *Bioinformation* **1**, 414 (2007).
15. B. A. Golomb, Acetylcholinesterase inhibitors and Gulf War illnesses. *Proc Natl Acad Sci U S A* **105**, 4295 (Mar 18, 2008).
16. H. K. Kang, B. Li, C. M. Mahan, S. A. Eisen, C. C. Engel, Health of US veterans of 1991 Gulf War: a follow-up survey in 10 years. *J Occup Environ Med* **51**, 401 (Apr, 2009).
17. L. Yang *et al.*, Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. *Reprod Toxicol* **28**, 245 (Sep, 2009).
18. C. Amourette *et al.*, Gulf War illness: Effects of repeated stress and pyridostigmine treatment on blood-brain barrier permeability and cholinesterase activity in rat brain. *Behav Brain Res* **203**, 207 (Nov 5, 2009).
19. W. Norton, L. Bally-Cuif, Adult zebrafish as a model organism for behavioural genetics. *BMC Neurosci* **11**, 90 (2010).
20. S. J. Schnorr, P. J. Steenbergen, M. K. Richardson, D. L. Champagne, Measuring thigmotaxis in larval zebrafish. *Behav Brain Res* **228**, 367 (Mar 17, 2012).
21. C. de Esch *et al.*, Locomotor activity assay in zebrafish larvae: influence of age, strain and ethanol. *Neurotoxicol Teratol* **34**, 425 (Jul, 2012).
22. C. Trapnell, L. Pachter, S. L. Salzberg, TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105 (May 1, 2009).
23. P. Cingolani *et al.*, Using Drosophila melanogaster as a Model for Genotoxic Chemical Mutational Studies with a New Program, SnpSift. *Front Genet* **3**, 35 (2012).
24. S. Anders, W. Huber, Differential expression analysis for sequence count data. *Genome Biol* **11**, R106 (2010).
25. M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139 (Jan 1, 2010).
26. J. Li, R. Tibshirani, Finding consistent patterns: A nonparametric approach for identifying differential expression in RNA-Seq data. *Stat Methods Med Res*, (Nov 28, 2011).
27. A. K. Wong *et al.*, IMP: a multi-species functional genomics portal for integration, visualization and prediction of protein functions and networks. *Nucleic Acids Res* **40**, W484 (Jul, 2012).
28. C. Y. Park *et al.*, Functional knowledge transfer for high-accuracy prediction of under-studied biological processes. *PLoS Comput Biol* **9**, e1002957 (2013).
29. V. Guryev *et al.*, Genetic variation in the zebrafish. *Genome Res* **16**, 491 (Apr, 2006).
30. Q. Xiong, N. Ancona, E. R. Hauser, S. Mukherjee, T. S. Furey, Integrating genetic and gene expression evidence into genome-wide association analysis of gene sets. *Genome Res* **22**, 386 (Feb, 2012).
31. I. Shaked, G. Zimmerman, H. Soreq, Stress-induced alternative splicing modulations in brain and periphery: acetylcholinesterase as a case study. *Ann N Y Acad Sci* **1148**, 269 (Dec, 2008).
32. D. Kaufer, A. Friedman, S. Seidman, H. Soreq, Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature* **393**, 373 (May 28, 1998).

33. B. Mauck, J. B. Lucot, S. Paton, R. D. Grubbs, Cholinesterase inhibitors and stress: effects on brain muscarinic receptor density in mice. *Neurotoxicology* **31**, 461 (Sep, 2010).
34. D. Alsop, M. M. Vijayan, Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am J Physiol Regul Integr Comp Physiol* **294**, R711 (Mar, 2008).
35. R. J. Egan *et al.*, Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav Brain Res* **205**, 38 (Dec 14, 2009).
36. G. B. Downes, M. Granato, Acetylcholinesterase function is dispensable for sensory neurite growth but is critical for neuromuscular synapse stability. *Dev Biol* **270**, 232 (Jun 1, 2004).
37. M. Granato *et al.*, Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123**, 399 (Dec, 1996).

APPENDIX

Personnel receiving pay from the research effort:

Richard Myers
Chris Gunter
Kelly Williams
Pamela Croom

Supporting Data (pg 1 of 6)

Functional annotation of DE genes for PB		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	0.63			
GOTERM_BP_FAT	GO:0043049~otic placode formation	4.20			
GOTERM_BP_FAT	GO:0045449~regulation of transcription	0.21			
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	0.75			
GOTERM_CC_FAT	GO:0005581~collagen	1.34			
GOTERM_CC_FAT	GO:0044420~extracellular matrix part	4.45			
GOTERM_MF_FAT	GO:0003677~DNA binding	0.04			
GOTERM_MF_FAT	GO:0003700~transcription factor activity	0.01			
GOTERM_MF_FAT	GO:0005201~extracellular matrix structural constituent	4.34			
GOTERM_MF_FAT	GO:0030528~transcription regulator activity	0.02			
INTERPRO	IPR000047:Helix-turn-helix motif, lambda-like repressor	3.16			
INTERPRO	IPR000885:Fibrillar collagen, C-terminal	0.28			
INTERPRO	IPR001356:Homeobox	0.10			
INTERPRO	IPR003070:Orphan nuclear receptor	1.00			
INTERPRO	IPR012287:Homeodomain-related	0.13			
INTERPRO	IPR017970:Homeobox, conserved site	1.21			
PIR_SUPERFAMILY	PIRSF002255:collagen alpha 1(I) chain	1.03			
SMART	SM00038:COLFI	0.34			
SMART	SM00389:HOX	0.34			
SP_PIR_KEYWORDS	developmental protein	0.05			
SP_PIR_KEYWORDS	DNA binding	2.15			
SP_PIR_KEYWORDS	dna-binding	<0.01			
SP_PIR_KEYWORDS	Homeobox	0.20			
SP_PIR_KEYWORDS	nucleus	0.23			
SP_PIR_KEYWORDS	transcription regulation	2.71			
UP_SEQ_FEATURE	DNA-binding region:Homeobox	0.06			
UP_SEQ_FEATURE	short sequence motif:PDZ-binding	3.30			
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism			<0.01	
GOTERM_BP_FAT	GO:0006835~dicarboxylic acid transport			4.60	
GOTERM_BP_FAT	GO:0008202~steroid metabolic process			3.12	
GOTERM_BP_FAT	GO:0016125~sterol metabolic process			1.21	
GOTERM_MF_FAT	GO:0005310~dicarboxylic acid transmembrane transporter activity			3.78	
GOTERM_MF_FAT	GO:0015370~solute:sodium symporter activity			1.48	
GOTERM_MF_FAT	GO:0017153~sodium:dicarboxylate symporter activity			2.74	
GOTERM_MF_FAT	GO:0020037~heme binding			0.14	
INTERPRO	IPR001128:Cytochrome P450			0.31	

Supporting Data (pg 2 of 6)

Functional annotation of DE genes for PB		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
INTERPRO	IPR001991:Sodium:dicarboxylate symporter			2.79	
INTERPRO	IPR006821:Intermediate filament, DNA-binding region			<0.01	
INTERPRO	IPR017972:Cytochrome P450, conserved site			0.20	
INTERPRO	IPR018107:Sodium:dicarboxylate symporter, conserved site			2.79	
KEGG_PATHWAY	dre00100:Steroid biosynthesis			<0.01	
KEGG_PATHWAY	dre00650:Butanoate metabolism			0.25	
SP_PIR_KEYWORDS	lipid synthesis			1.94	
SP_PIR_KEYWORDS	oxidoreductase			0.05	
SP_PIR_KEYWORDS	sterol biosynthesis			3.01	
GOTERM_BP_FAT	GO:0007398~ectoderm development				0.63
GOTERM_CC_FAT	GO:0005911~cell-cell junction				<0.01
GOTERM_CC_FAT	GO:0016327~apicolateral plasma membrane				1.75
GOTERM_CC_FAT	GO:0030054~cell junction				<0.01
GOTERM_CC_FAT	GO:0043296~apical junction complex				1.75
GOTERM_MF_FAT	GO:0004175~endopeptidase activity				1.18
GOTERM_MF_FAT	GO:0008233~peptidase activity				2.73
INTERPRO	IPR000033:Low-density lipoprotein receptor, YWTD repeat				3.94
INTERPRO	IPR002172:Low density lipoprotein-receptor, class A, cysteine-rich				0.04
SMART	SM00192:LDLa				0.06
SP_PIR_KEYWORDS	Protease				0.17

Functional annotation of DE genes for stress		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism	4.73			
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	0.93			
GOTERM_BP_FAT	GO:0045449~regulation of transcription	1.41			
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	1.07			
GOTERM_MF_FAT	GO:0003677~DNA binding	1.48			
GOTERM_MF_FAT	GO:0003774~motor activity	0.09			
GOTERM_MF_FAT	GO:0005506~iron ion binding	4.12			
GOTERM_MF_FAT	GO:0016714~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced pteridine as one donor, and incorporation of one atom of oxygen	2.58			
GOTERM_MF_FAT	GO:0030528~transcription regulator activity	0.04			
GOTERM_MF_FAT	GO:0043565~sequence-specific DNA binding	0.06			
INTERPRO	IPR001273:Aromatic amino acid hydroxylase	2.00			

Supporting Data (pg 3 of 6)

Functional annotation of DE genes for stress		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
INTERPRO	IPR018301:Aromatic amino acid hydroxylase, iron/copper binding site	2.00			
INTERPRO	IPR019773:Tyrosine 3-monooxygenase-like	2.00			
INTERPRO	IPR019774:Aromatic amino acid hydroxylase, C-terminal	2.00			
PIR_SUPERFAMILY	PIRSF000336:TH	1.73			
SP_PIR_KEYWORDS	nucleus	2.20			
SP_PIR_KEYWORDS	Transcription	1.88			
SP_PIR_KEYWORDS	transcription regulation	0.67			
INTERPRO	IPR017995:Homeobox protein, antennapedia type		<0.01		
UP_SEQ_FEATURE	short sequence motif:Antp-type hexapeptide		0.01		
GOTERM_BP_FAT	GO:0003012~muscle system process			0.01	
GOTERM_BP_FAT	GO:0006936~muscle contraction			0.01	
GOTERM_BP_FAT	GO:0006941~striated muscle contraction			0.01	
GOTERM_CC_FAT	GO:0005856~cytoskeleton			0.04	
GOTERM_CC_FAT	GO:0005859~muscle myosin complex			<0.01	
GOTERM_CC_FAT	GO:0005863~striated muscle thick filament			<0.01	
GOTERM_CC_FAT	GO:0015629~actin cytoskeleton			1.24	
GOTERM_CC_FAT	GO:0016459~myosin complex			0.18	
GOTERM_CC_FAT	GO:0016460~myosin II complex			<0.01	
GOTERM_CC_FAT	GO:0030016~myofibril			0.06	
GOTERM_CC_FAT	GO:0030017~sarcomere			0.06	
GOTERM_CC_FAT	GO:0032982~myosin filament			<0.01	
GOTERM_CC_FAT	GO:0043292~contractile fiber			0.06	
GOTERM_CC_FAT	GO:0044430~cytoskeletal part			0.03	
GOTERM_CC_FAT	GO:0044449~contractile fiber part			0.06	
GOTERM_MF_FAT	GO:0003779~actin binding			0.82	
GOTERM_MF_FAT	GO:0008092~cytoskeletal protein binding			2.51	
INTERPRO	IPR000048:IQ calmodulin-binding region			0.05	
INTERPRO	IPR001609:Myosin head, motor region			0.07	
INTERPRO	IPR001978:Troponin			0.03	
INTERPRO	IPR002928:Myosin tail			0.01	
INTERPRO	IPR004009:Myosin, N-terminal, SH3-like			0.01	
INTERPRO	IPR015650:Heavy chain of Myosin			<0.01	
SMART	SM00015:IQ			0.02	
SMART	SM00242:MYSc			0.04	
SP_PIR_KEYWORDS	actin-binding			0.04	

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Functional annotation of DE genes for stress		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
SP_PIR_KEYWORDS	coiled coil			0.42	
SP_PIR_KEYWORDS	motor protein			0.04	
SP_PIR_KEYWORDS	muscle protein			0.01	
SP_PIR_KEYWORDS	myosin			0.09	
SP_PIR_KEYWORDS	thick filament			<0.01	
SMART	SM00338:BRLZ				3.30
Functional annotation of DE genes for PB x stress interaction		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
GOTERM_BP_FAT	GO:0008283~cell proliferation	3.09			
INTERPRO	IPR001699:Transcription factor, T-box	2.19			
INTERPRO	IPR018186:Transcription factor, T-box, conserved site	2.19			
SMART	SM00425:TBOX	2.72			
GOTERM_BP_FAT	GO:0006350~transcription		3.66		
GOTERM_MF_FAT	GO:0004703~G-protein coupled receptor kinase activity		3.84		
GOTERM_MF_FAT	GO:0050254~rhodopsin kinase activity		1.58		
INTERPRO	IPR000239:GPCR kinase		3.42		
INTERPRO	IPR001827:Homeobox protein, antennapedia type, conserved site		<0.01		
PIR_SUPERFAMILY	PIRSF000584:G protein-coupled receptor kinase		3.23		
PIR_SUPERFAMILY	PIRSF002612:homeotic protein Hox A5/D4		<0.01		
PIR_SUPERFAMILY	PIRSF500608:homeotic protein Hox A5		1.00		
UP_SEQ_FEATURE	short sequence motif:Antp-type hexapeptide		<0.01		
INTERPRO	IPR001483:Urotensin II			3.96	
PIR_SUPERFAMILY	PIRSF001793:urotensin II			3.52	
GOTERM_BP_FAT	GO:0006694~steroid biosynthetic process				2.52
GOTERM_BP_FAT	GO:0006811~ion transport				<0.01
GOTERM_BP_FAT	GO:0006812~cation transport				<0.01
GOTERM_BP_FAT	GO:0006813~potassium ion transport				3.51
GOTERM_BP_FAT	GO:0006820~anion transport				1.51
GOTERM_BP_FAT	GO:0006836~neurotransmitter transport				3.16
GOTERM_BP_FAT	GO:0008202~steroid metabolic process				1.13
GOTERM_BP_FAT	GO:0008610~lipid biosynthetic process				3.33
GOTERM_BP_FAT	GO:0015672~monovalent inorganic cation transport				<0.01
GOTERM_BP_FAT	GO:0015698~inorganic anion transport				3.62
GOTERM_BP_FAT	GO:0016125~sterol metabolic process				0.19
GOTERM_BP_FAT	GO:0016126~sterol biosynthetic process				0.43
GOTERM_BP_FAT	GO:0030001~metal ion transport				<0.01

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Functional annotation of DE genes for PB x stress interaction		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
GOTERM_CC_FAT	GO:0005887~integral to plasma membrane				0.27
GOTERM_CC_FAT	GO:0008021~synaptic vesicle				<0.01
GOTERM_CC_FAT	GO:0008076~voltage-gated potassium channel complex				4.98
GOTERM_CC_FAT	GO:0016021~integral to membrane				2.90
GOTERM_CC_FAT	GO:0016023~cytoplasmic membrane-bounded vesicle				1.91
GOTERM_CC_FAT	GO:0030135~coated vesicle				0.38
GOTERM_CC_FAT	GO:0030136~clathrin-coated vesicle				0.08
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane				1.55
GOTERM_CC_FAT	GO:0031226~intrinsic to plasma membrane				0.47
GOTERM_CC_FAT	GO:0031410~cytoplasmic vesicle				4.68
GOTERM_CC_FAT	GO:0031988~membrane-bounded vesicle				1.91
GOTERM_CC_FAT	GO:0034702~ion channel complex				0.04
GOTERM_CC_FAT	GO:0034703~cation channel complex				0.02
GOTERM_CC_FAT	GO:0034705~potassium channel complex				4.98
GOTERM_CC_FAT	GO:0044456~synapse part				<0.01
GOTERM_CC_FAT	GO:0045202~synapse				<0.01
GOTERM_MF_FAT	GO:0005216~ion channel activity				<0.01
GOTERM_MF_FAT	GO:0005230~extracellular ligand-gated ion channel activity				2.60
GOTERM_MF_FAT	GO:0005244~voltage-gated ion channel activity				0.03
GOTERM_MF_FAT	GO:0005261~cation channel activity				<0.01
GOTERM_MF_FAT	GO:0015267~channel activity				<0.01
GOTERM_MF_FAT	GO:0015293~symporter activity				0.47
GOTERM_MF_FAT	GO:0022803~passive transmembrane transporter activity				<0.01
GOTERM_MF_FAT	GO:0022832~voltage-gated channel activity				0.04
GOTERM_MF_FAT	GO:0022836~gated channel activity				<0.01
GOTERM_MF_FAT	GO:0022838~substrate specific channel activity				<0.01
GOTERM_MF_FAT	GO:0022843~voltage-gated cation channel activity				0.06
GOTERM_MF_FAT	GO:0030955~potassium ion binding				0.23
GOTERM_MF_FAT	GO:0031420~alkali metal ion binding				0.03
GOTERM_MF_FAT	GO:0046873~metal ion transmembrane transporter activity				<0.01
INTERPRO	IPR000532:Glucagon/GIP/secretin/VIP				1.00
INTERPRO	IPR001664:Intermediate filament protein				<0.01
INTERPRO	IPR003091:Voltage-dependent potassium channel				3.68
INTERPRO	IPR004031:PMP-22/EMP/MP20/Claudin				1.99
INTERPRO	IPR005821:Ion transport				0.39
INTERPRO	IPR006821:Intermediate filament, DNA-binding region				0.09

Supporting Data (pg 6 of 6)

Functional annotation of DE genes for PB x stress interaction		Brain		Muscle	
Category	Term	0-wk	8-wk	0-wk	8-wk
INTERPRO	IPR017995:Homeobox protein, antennapedia type				<0.01
KEGG_PATHWAY	dre00100:Steroid biosynthesis				<0.01
SMART	SM00070:GLUCA				0.55
SP_PIR_KEYWORDS	cell junction				0.06
SP_PIR_KEYWORDS	duplication				4.66
SP_PIR_KEYWORDS	ion transport				<0.01
SP_PIR_KEYWORDS	ionic channel				<0.01
SP_PIR_KEYWORDS	iron				3.39
SP_PIR_KEYWORDS	lipid synthesis				0.09
SP_PIR_KEYWORDS	membrane				0.22
SP_PIR_KEYWORDS	potassium				0.76
SP_PIR_KEYWORDS	potassium transport				1.12
SP_PIR_KEYWORDS	Sodium transport				0.04
SP_PIR_KEYWORDS	Steroid biosynthesis				0.23
SP_PIR_KEYWORDS	sterol biosynthesis				0.07
SP_PIR_KEYWORDS	Symport				2.81
SP_PIR_KEYWORDS	synapse				0.50
SP_PIR_KEYWORDS	Transcription				0.89
SP_PIR_KEYWORDS	transcription regulation				1.01
SP_PIR_KEYWORDS	transmembrane				0.01
SP_PIR_KEYWORDS	transport				1.02
SP_PIR_KEYWORDS	voltage-gated channel				3.66